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Mining for Biological Control Agents against B. glumae, the Causal Agent of Bacterial Panicle Blight of Rice.

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Mining for Biological Control Agents against *B. glumae*,
the Causal Agent of Bacterial Panicle Blight of Rice.

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Abstract

Burkholderia glumae is the causal agent of the emerging disease, Bacterial Panicle Blight of rice, a serious disease that can significantly decrease yield and poses a threat to rice production worldwide. This thesis is concerned with searching for a biological control agent to control this disease. Plant associated microbes are a good source of beneficial bacteria which can be exploited for use as a biological control agent. It is possible that the microbiomes of cultivars which are known to be more resistant to plant pathogens may contain more microbes which inhibit those pathogens and therefore could be used as biological control agents in agriculture. This thesis can be divided into two parts, part one which is concerned with identifying inhibitory bacteria from the moderately resistant rice cultivar Jupiter and from the Rojas Lab collection, and part two which is concerned with identifying the mode of control these inhibitory bacteria use against *B. glumae*. The hypotheses for part one are that there are different rice associated bacteria present on the susceptible cultivar Bengal and the moderately resistant cultivar Jupiter, and that the rice associated bacteria unique to Jupiter add to its resistance to the pathogen. The hypotheses for part two are that the inhibitory bacteria identified in part one use either competition or antibiosis to inhibit *B. glumae*. These hypotheses were tested using a variety of experiments including microbiome isolation, inhibition assays, plant inoculations and inhibitory compound isolation experiments to find bacteria which could control *B. glumae*, and to discover their mechanisms of inhibition. Though the bacteria isolated from Bengal and Jupiter were comprised of different bacterial strains, the Jupiter-specific bacteria were not particularly good at inhibiting the growth of the pathogen. Two strains of bacteria from the Rojas lab collection, *Burkholderia cenocepacia* and *Pseudomonas fluorsecens*, however were found to be very successful at inhibiting the growth of *B. glumae* and successfully reduced symptoms of infection in vivo. Furthermore it was found that these two bacterial strains do in fact control *B. glumae* through the production of inhibitory compounds.

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Introduction

Burkholderia glumae is the bacterial pathogen responsible for the disease Bacterial Panicle Blight of rice. Symptoms of this disease include seedling stunting and chlorosis, discoloration of the rice spikelets, rice panicles not filling resulting in decreased yield, and discoloration of the sheath on infected plants (Iiyama 1995; Nandakumar et al., 2009; Wamishe et al., 2015). This disease is favored by hot humid weather, specifically hot summer nights, which makes it a concerning emerging disease due to global warming (Wamishe et al., 2015; Ham et al., 2011; Mizobuchi et al., 2016).

The methods of control for this pathogen in the United States are currently limited. There are no completely resistant cultivars of rice to the disease (Mizobuchi et al., 2016). Cultural control methods include early planting to plants flowering during hot summer nights which favor the disease (Wamishe et al., 2015). Chemical control methods include the seed or foliar spray treatments of the quinolone antibiotic oxolinic acid which is commonly used in Asia, but is not approved for use in the United States (Hikichi et al., 1993; Maeda et al., 2004). With few methods of control available to growers in the United States, this research aims to look at potential biological control agents against the disease.

Biological control agents isolated from rice associated bacteria have been shown to have some success in suppressing the pathogen (Shrestha et al., 2016). Though those results were not reproducible, this research intends to expand upon it and search for more bacterial strains which suppress the pathogen. In this thesis we explored two sources for potential biological control agents, firstly the bacteria associated with the moderately resistant rice cultivar Jupiter, and secondly bacteria from the Rojas Lab collection. Bacterial strains from moderately resistant rice cultivar Jupiter and susceptible rice cultivar Bengal were isolated and compared with the hypothesis that rice cultivars with different susceptibilities to *B. glumae* infection would

also have different rice associated bacterial compositions. Bacteria isolated from Jupiter alone were then tested for their suppressive activity against *B. glumae* with the hypothesis that bacteria present on Jupiter and not Bengal were partially responsible for Jupiter's moderate resistance to the disease. The second source mined for potential biological control agents was the Rojas Lab collection.

After bacteria with antagonistic activity against *B. glumae* were identified their mechanisms of control were explored. Based on the results of our experiments two main methods of control were explored, antibiosis, or the production of inhibitory compounds, and competition, or the ability of one bacterial strain to grow more quickly and overtake the other, with the hypotheses being that the potential biological control bacteria were using antibiosis or competition as their main mechanism to control *B. glumae*.

Chapter 1. Literature Review

1.1. Bacterial Panicle blight of rice

Bacterial panicle blight of rice is caused by two *Burkholderia* species*: Burkholderia glumae* and *B. gladioli,* with *B. glumae* being more prevalent and aggressive compared to *B. gladioli* (Fory et al., 2014; Nandakumar and Rush, 2007; Nandakumar et al., 2009). This disease is distributed worldwide, and in the United States has caused severe losses in Arkansas, Texas, Mississippi and Louisiana (Nandakumar et al., 2009). The disease is also known under the name bacterial seedling rot and bacterial grain rot (Goto and Ohata, 1956). *B. glumae* and *B. gladioli* can grow at temperatures higher than 40^{o}C; warmer than the optimal growth temperature for most plant pathogenic bacteria (Nandakumar et al., 2009). Hot and humid weather leads to severe *B. glumae* infection in the field (Wamishe et al., 2015) and, consequently, the most devastating effects of the disease were observed in 2010, when temperatures during the growing season were higher than average especially at night. Consequently, these pathogens have the potential to become more problematic with the continued rise of global temperature (Ham et al., 2011; Mizobuchi et al., 2016). The mechanisms by which *B. glumae* is transmitted and causes infection are not fully understood. However, the availability of genomic sequences for several strains (Lim et al., 2009; Knapp et al., 2015; Johnson et al., 2015) and increased interest in investigating these pathogens, will provide further understanding of the disease to guide efforts to control it or decrease losses in rice production.

1.2. Symptoms

Although the disease cycle is not fully understood, it is known that transmission of the pathogen occurs through contaminated seeds. Upon seed germination, bacteria start an epiphytic lifestyle and further migrate to different parts of the plant (Li et al., 2016) where it causes disease

symptoms in sheath and panicles. Symptoms on infected seedlings include stunting and chlorosis (Iiyama, 1995). In older plants, symptoms in vegetative tissue are characterized by gray lesions surrounded by brown margins (Nandakumar et al., 2009). During the reproductive stages, bacteria infect reproductive tissues in the panicle interfering with grain development. Symptoms in kernels start with a brown margin at their base with the branches of the panicle remaining green (Nandakumar, 2009; Wamishe et al., 2015). At later stages, the entire panicle turns light brown with most of the kernels unfilled; as a result, the panicles remain erect, in contrast with healthy plants where the weight of the grains causes panicle bending (Nandakumar, 2009; Wamishe et al., 2015).

1.3. Mechanisms of pathogenicity

B. glumae produces several virulence factors that are likely required at different stages of the disease. The main virulence factor, responsible for the symptoms is the toxin "Toxoflavin". Toxoflavin toxicity is due to generation of hydrogen peroxide (Latuasan and Berends, 1961) which causes cell death and subsequent chlorosis and stunting in seedlings, sprouts and roots and symptoms of blight in maturing panicles (Sato et al., 1989; Iiyama et al., 1995). Disease is almost always associated with *B. glumae* strains that produce toxoflavin (Iiyama et al., 1995; Nandakumar et al., 2009; Karki et al,.2012). Toxoflavin biosynthesis requires the activities of the biosynthetic genes *toxA, toxB, toxC, toxD* and *toxE* that are clustered in an operon (Suzuki et al., 2004; Kim et al.,2004), whereas toxoflavin transport requires genes *toxF, toxG, toxH* and *toxI* (Kim et al., 2004). Both operons are regulated by the regulatory proteins ToxJ and ToxR, which in turn are upregulated by TofR (Kim et al, 2004).

Other virulence factors have been identified based on the reduced virulence of mutants harboring mutations in specific genes, indicating that those genes encode virulence factors. However, it is not clear how these virulence factors contribute to pathogenesis. Based on analysis of the sequenced genomes, *B. glumae* encode type II and type III secretion systems (Lim et al.,

2009; Knapp et al., 2016; Johnson et al., 2015; Fory et al., 2014), and using a proteomics approach 46 proteins were found to be secreted as part of the type three secretion system (Kang et al., 2009). Additional virulence factors produced by *B. glumae* and likely secreted in a type II-dependent manner include a lipase (Devescovi et al., 2007) and two endopolygalaturonases, PehA and PehB (Degrassi et al., 2008). PehA and PehB were shown to have enzymatic activity *in vitro,* but their assumed function in the pathogenicity of *B. glumae* was not fully demonstrated (Degrassi et al., 2008).

The type III secretion system is functional as demonstrated by its ability to elicit a hypersensitive response in tobacco (Kang et al.,2008) and to deliver heterologous proteins to plants (Sharma et al,. 2013). Only three putative effectors for the *B. glumae* type III secretion system have been identified: HrpK1, Eop3 and HrpW, either by proteomic analysis or by sequence comparisons with effectors of other plant pathogenic bacteria (Kang et al., 2008; Fory et al., 2014). Because identifying bacterial effectors based on sequence motifs is inherently difficult due to complex rules associated with their amino acid sequences (Petnicki-Ocwieja et al., 2002), other possible effectors encoded in the *B. glumae* genome remain to be identified. Although more investigations are needed to fully understand the function of the secretion systems in *B. glumae,* it is clear that they contribute to virulence as mutants defective in either system showed reduced ability to grow *in planta* and reduced virulence in panicles, even though they were able to produce normal amounts of toxoflavin (Kang et al., 2008).

1.4. Regulation of virulence

All the virulence factors implicated in *B. glumae* pathogenesis are regulated by quorum sensing, a mechanism of bacterial communication that activates gene expression in a population-density dependent manner (Vadakkan et al., 2018). Bacterial communication is mediated by signaling molecules called autoinducers (Vadakkan et al., 2018). In *B. glumae,* quorum sensing is regulated by a LuxI/LuxR- type quorum sensing system, wherein the *LuxI* ortholog *TofI* encodes

the N-acyl-homoserine lactone synthase that synthesizes two autoinducers: N-octanoyl homoserine lactone (C8-HSL) and N-hexanoyl homoserine lactone (C6-HSL) (Kim et al, 2004). The *LuxR*- ortholog *TofR* encodes the transcriptional regulator that regulate the expression of target genes containing *lux* box-like promoter sequences upon binding C8-HSL (Kim et al, 2004). The *B. glumae* quorum sensing system is essential for pathogenicity as, quorum sensing mutants have significant reduction in virulence (Devescovi et al, 2007).

1.5. Disease Management

1.5.1. Chemical Control

The quinolone antibiotic oxolinic acid, used as seed treatment and as foliar sprays, is the only available chemical treatment to control bacterial panicle blight was widely used in Asia (Hikichi et al., 1993, Maeda et al., 2004), but is not approved for use in the United States. Oxolinic acid enhances DNA supercoiling by targeting the DNA gyrase gene *GyrA*, which is responsible for bacterial DNA winding and unwinding. Increase in supercoiling inhibits DNA synthesis (Franco and Drlica, 1989). However, the identification of several *B. glumae* strains resistant to oxolinic acid has limited its use (Maeda et al., 2004).

Alternative methods of chemical control could include using compounds that interfere with quorum sensing such as those inhibiting the synthesis of the autoinducer (Chung et al., 2011). However, there is no evidence of any further testing to determine if they are effective reducing or eliminating the disease.

1.5.2. Cultural Practices

BGRcast is a forecast system used in Korea that uses temperature and humidity to calculate the likelihood of a bacterial panicle blight epidemic, and makes recommendations for oxolinic acid application to prevent an epidemic (Lee et al., 2015). This system has an accuracy rate of 71.4% (Lee et al., 2015). Other cultural practices including planting pathogen-free seed

can help reduce the amount of inoculum present in a field as the pathogen can be seed borne (Wamishe et al., 2015). Early planting can also help reduce the severity of disease as has been observed in the United States when planting from late March to mid-April versus planting in May as has been the common practice (Wamishe et al., 2015). This is likely effective because the rice planted earlier in the season flowers earlier and therefore escapes the hottest part of the summer.

1.5.3. Resistant rice cultivars

There are no rice cultivars that show complete resistance to *B. glumae*, however, there are several cultivars with partial resistance (Mizobuchi et al., 2016). Identifying sources of resistance has been challenging because different methods of inoculation produce different results (Mizobuchi et al., 2016). One QTL has been located for resistance to bacterial seedling rot caused by *B. glumae* (qRBS1) which is located in a 393-kb interval of the short arm of chromosome 10 in Niponbare (Mizobuchi et al., 2013). There are twelve more QTLs for bacterial grain rot associated with resistance to *B. glumae* located across seven different rice chromosomes (Mizobuchi et al., 2016).

High levels of resistance to *B. glumae* have been achieved by generating transgenic rice lines overexpressing BSR1 (Broad-Spectrum Resistance 1), a gene encoding a receptor-like kinase that confers resistance to two rice diseases: blast (*Magnaporthe oryzae*) and leaf blight (*Xanthomonas oryzae pv. oryzae*) (Debouzet et al., 2011; Maeda et al., 2016).

This demonstrates that different rice genotypes contain resistance genes effective against *B. glumae* that could be used to develop completely resistant cultivars in the future.

1.5.4. Biological Control

Several efforts have been initiated to identify sources of biological control against *B. glumae.* For example, *Paenibacillus polymyxa* JH2 is an environmental bacteria that produces a toxoflavin-degrading enzyme (Jung et al, 2011). However, no follow-up studies have been

conducted to determine if this bacteria can be an effective biological control agent against *B glumae.* In a more recent study, a metagenomics library was used to isolate *E. coli* mutants that were able to grow in the presence of toxoflavin, and identified a toxoflavin-degrading enzyme, TxeA (Choi et al., 2018). Therefore, this enzyme could potentially be used to generate transgenic plants that could degrade the bacterial toxin or, to engineer antagonistic bacteria that could compete with *B. glumae.*

Another approach aimed at identifying potential biological control agents against *B. glumae* consisted of isolating rice-associated bacteria and further evaluating whether they could inhibit *B. glumae* growth *in vitro* (Shrestha at al., 2016). Using this approach, twenty nine bacterial strains from the genera *Bacillus* and *Lysinibacillus* were identified that showed inhibitory activity against *B. glumae in vitro.* Five of the *Bacillus* strains were further used in field experiments wherein rice plants were pre-treated with such strains before inoculation with *B. glumae*. Pre-treatment resulted in reduced disease severity. However, these results were not reproducible in subsequent years (Shrestha et al., 2016), limiting the value of the results found *in vitro*.

2. Justification and objectives

Because of the lack of effective methods to control Bacterial Panicle Blight, new research is needed to develop disease control methods. Although some genes have been identified conferring resistance against *B. glumae,* there is still limited information on them to make them useful for conventional breeding or transgenic approaches. Moreover, the mechanisms of pathogenicity in *B. glumae* are not fully understood and therefore, designing targeted approaches to control the disease are not feasible at the moment. However, because previous work investigating the rice-associated bacteria identified bacterial strains capable to inhibiting the growth of *B. glumae in vitro* (Shrestha at al., 2016), that constitutes evidence that using similar approaches could yield the identification of potentially biological control agents with activity *in vitro* and *in planta.* Therefore, one of the objectives of this study is to analyze the rice-associated

microbial populations to identify biological control agents against *B. glumae.* Because previous work has shown that one of the factors that influences the composition of the plant-associated microbes is the genotype of the plant (Rossman et al., 2017), one of the hypothesis of this study is that different rice genotypes have different microbial composition associated with them. The second hypothesis is that resistant rice cultivars harbor microorganisms that can suppress the growth of *B. glumae*. Identifying suppressing, culturable, bacteria would enable their use as biological control agents. Identification of biological control agents against *B. glumae* will lead to the characterization of their mechanism of action, a second objective of this work.

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Chapter 2: Identifying rice-associated bacteria specific to a resistant rice cultivar *as* potential biological control agents against *Burkholderia glumae*

Introduction

Microorganisms in the environment establish beneficial and pathogenic interactions with eukaryotes. One of those interactions is commensalism, where one of the organisms benefit, but there is no benefit or penalty for the other. Commensal microbial populations can affect the health of the organisms they are associated with. For example, commensal microbes associated with the human body, have been linked to non-infectious diseases such as obesity, inflammatory bowel disease, cardiovascular disease, colon cancer, rheumatoid arthritis and dental diseases, among others (Gilbert et al., 2016).

Similar to the contribution of commensal microorganisms to human health, plant-associated microorganisms, usually in commensalistic relationships, can contribute to plant health in different ways: increasing nutrient acquisition, providing hormones for plant growth and preventing infectious diseases either indirectly, by inducing defense responses or directly, by interacting with plant pathogens (Berg et al., 2009; Bakker et al., 2013). Root-associated microorganisms in the rhizosphere are responsible for the occurrence of "suppressive soils", in which a given pathogen, although present, is unable to cause disease (Baker and Cook, 1974), like the suppression of "Take-all" (Weller, 2007), "Fusarium wilt" (Alabouvette, 1999) and "Tobacco black rot" (Kyselkova et al., 2009). The evidence that the soil microbial populations are responsible for suppressive soils has been provided by experiments showing that soil pasteurization decreases suppressive properties, whereas addition of organic amendments that support microbial populations increase disease suppression (Mendes et al., 2011). Whereas the individual microorganisms that contribute to this phenomenon have not been characterized, it has been proposed that plant-associated microorganisms suppress pathogens by competing

with them for nutrients or by secreting compounds that inhibit pathogen growth (Berendsen et al., 2012).Thus, identification of plant associated microbes that negatively impact a given pathogen could provide environmentally conscious alternatives to chemical control (Berg et al., 2009).

High throughput technologies investigating the microbial composition in plants, have revealed connections between the composition of the microbial populations, the plant environment, type of tissue and genotype (Muller et al., 2016). In the case of rice, the microbial communities associated with roots and leaves have been investigated by next generation sequencing approaches (Sessitsch et al., 2012; Knief et al., 2012; Edwards et al., 2015), and similar studies although in smaller scale, have used rice-associated microorganisms to control fungal rice diseases such as Rice blast (Spence et al., 2014) and Sheath blight (Kanjanamaneesathian et al.,1998; Kazempour, 2004; Shrestha et al., 2016; Singh et al.,2016). Moreover, rice-associated bacteria was previously investigated as potential biological control agents against *B. glumae* (Shrestha et al., 2016), and that work successfully identified twenty nine bacterial strains from the genera *Bacillus* and *Lysinibacillus* that showed inhibitory activity against *B. glumae in vitro,* being the most effective strains of *B. subtilis, B. methylotrophicus and B. amyloliquefaciens*. However, these strains were not consistently effective under field conditions (Shrestha et al., 2016).

The main objective of this study is to characterize the bacterial populations between two rice genotypes exhibiting differential responses against *Burkholderia glumae,* one being moderately resistant (cultivar Jupiter) and the other being susceptible (cultivar Bengal). The hypothesis of this study is that rice genotypes that are cultivar Jupiter contains a microbial composition that inhibits *B. glumae* growth or its pathogenic activities. To test this hypothesis, bacteria specifically found in the Jupiter will be further tested to identify strains with antagonistic

activity against *B. glumae.* The second objective is to prospect from bacterial strains with antagonistic activity against *B. glumae* using the Rojas laboratory culture collection that includes bacteria previously isolated from the field. Identification of bacteria with antagonistic activity will further facilitate their use as biological control agents. Identification of biological control agents will facilitate further studies to test their efficacy in greenhouse conditions and eventually, field assays.

Materials and Methods

Bacterial strains

Three strains of *B. glumae* (UAPB10, UAPB11, UAPB12 and UAPB13) were obtained from Dr. Yeshi Wamishe (University of Arkansas Rice Research and Extension Center, Stuttgart AR). Other bacterial strains used were from the Rojas lab collection. Bacterial strains and their origins are listed in Table 3. Bacterial strains were retrieved from glycerol stocks kept at -80^{o}C, streaked on King's B (KB) agar and incubated at 30^{o}C for 18 h. Individual colonies were then cultured in 5mL KB broth overnight in a 30^{o}C shaker. These cultures were then centrifuged at 6,000rpm for 10 minutes and washed with sterile water three times. Bacterial concentration was measured using a spectrophotometer and bacterial inoculum were diluted in water to different concentrations depending on the experiment**.**

Rice cultivars

Rice cultivars Jupiter and Bengal are medium grain japonica that are moderately resistant and susceptible, respectively to *B. glumae*. Cultivar Wells is a long grain indica cultivar that is highly susceptible to *B. glumae.* Seeds from these cultivars were obtained from Dr. Yeshi Wamishe (Rice Research and Extension, Stuttgart, AR).

Isolation of bacteria associated with seeds and leaves

To isolate bacterial populations from seeds, 5g of seeds (with husks on) from each genotype were ground using mortars and pestles in sterile 1 x phosphate buffered-saline (PBS), pH 7.4. Ground seeds were added to 50 ml of sterile 1 x PBS, pH 7.4 and incubated for 2h at 4^{o}C. Solution containing ground seeds were serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in 1ml sterile water, plated on King's B (KB) agar and incubated at 30^{o}C for 24 h to retrieve bacterial populations.

To isolate bacterial populations from leaves, 20 seeds from each genotype were de-husked and sterilized in 70% bleach solution for five minutes. The seeds were then placed in sterile LC1 (Sunshine- Sun Gro) soil mix, covered with a thin layer of soil and grown for two weeks. After two weeks, four seedlings were transplanted to larger pots containing sterile soil. Plants were sprayed with ferrous sulfate every two days and fertilized every two weeks. At eight weeks, mature leaves were harvested and weighted, and five grams were cut into one-inch-long sections, ground in sterile 1 x PBS, pH 7.4 and added to 50 mL of sterile 1x PBS. pH 7.4 and incubated for 2h at 4^{o}C. Solution containing ground leaves were serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, plated on KB agar and incubated at 30^{o}C for 24h to retrieve bacterial populations.

Identification of bacterial strains associated with rice seeds and leaves

To characterize the culturable bacteria associated with seeds and leaves in 'Jupiter' and 'Bengal', twenty colonies were randomly selected from the dilution plates of 10^{-3} containing bacterial colonies clearly separated from others. Individual colonies were grown in 500 µL of Luria Bertani (LB) broth overnight for genomic DNA extraction. Overnight cultures were centrifuged for 5 min at 13,000rpm and pellets were resuspended in 500 µL of STE (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA**)** and 40 µL of 10% SDS. Samples were boiled for 5 min and then cooled on ice for 5 min. Samples were centrifuged for 5 min at 13,000 rpm

and 500µL of the supernatants were transferred to new tubes. DNA was precipitated with 250µL of isopropanol and incubated for 5 min at room temperature. DNA was collected by centrifugation for 5 min at 13,000rpm. Pellets were washed with 500µL of 70% ethanol, allowed to dry and then resuspended in 500µL sterile water.

To identify bacterial strains the 16S rRNA gene was amplified by PCR using bacterial genomic DNA as template and with the 16S rRNA primers: 27F 5' AGAGTTTGATCCTGGCTCAG 3' and 1492R 5' GGTTACCTTGTTACGACTT 3'. The PCR was performed using the following conditions: denaturation at 98^{o}C for 1:00 min, followed by 40 cycles of 98^{o}C for 10 sec, 45^{o}C for 30 sec 60^{o}C for 2:00 min) and final extension at 72^{o}C for 10:00 min. PCR products were run on a 1% agarose gel with Gel Red® at 100V for one hour. Amplicons were sequenced at Eton Bioscience (San Diego, CA) using as sequencing primers the 16S rRNA primers 27F and 1492R. Full length 16S rRNA sequences were assembled using SeqMan (DNAStar) (Madison, WI) and bacteria were identified by comparing the sequence with bacterial sequences available in the National Center for Biotechnology Information (NCBI) database, using BLASTN

Screening for antagonistic activity against *B. glumae*

Bacterial strains isolated from 'Jupiter' as well as bacteria available in the Rojas lab collection were used in a growth inhibition assay. For that purpose, overnight cultures of *B. glumae* strains UAPB10, 11 and 13 were washed in sterile water and the bacterial concentration measured. Prepared bacteria were mixed with molten KB agar maintained at 50^{o}C to a final concentration of OD_{600}= 0.01 ($1x10^{6}$ CFU/mL) and poured into sterile Petri plates. Five 6mm diameter sterile filter paper disks were added to solidified plates.

Bacterial strains isolated from 'Jupiter' and bacteria from the Rojas lab collection were prepared as described above and diluted to OD600= 0.01 or OD_{600}= 1 ($1X10^{8}$CFU/mL), depending on the assay. Five microliters of bacteria were added to four of the filter disks on the

KB agar containing *B. glumae*. The fifth disk was used as negative control with 5 µl sterile water. Plates were incubated at 30ºC for 48h. Growth inhibition of *B. glumae*, visualized as a halo around the filter disks containing bacteria, was assessed after two days. The diameter of the zone of inhibition was measured and used to calculate the area of the zone of inhibition to which it was subtracted the area of the filter paper disk. Average area of the zone of inhibition was calculated from the triplicates by determining the area of the zone of inhibition and subtracting the area of the filter paper disk. The experiment was repeated three times.

Pathogenicity assays

To evaluate the effect of antagonistic bacteria on disease development, *B. glumae* alone, or in combination with potentially antagonistic bacteria, was inoculated into eight- week-old rice plants by sheath injection and three- month- old flowering rice plants by panicle dip inoculation. For these experiments rice plants from genotype Wells were used as this cultivar is more susceptible to *B. glumae* than Bengal and its growth was more even under greenhouse conditions in comparison with the growth of Bengal. For sheath injection, overnight cultures of *B. glumae* and the antagonistic bacteria *B. cenocepacia* and *P. fluorescens* were diluted to OD_{600}= 0.125 ($1X10^8$ CFU/mL). Twenty microliters of *B. glumae* inoculum alone, or in combination with either *B. cenocepacia*, *P. fluorescens* or *E. coli* at 1:1 ratios were injected into the sheath of eight-week old plants from susceptible genotype 'Wells' using insulin syringes in triplicates. Plants injected with water were used as negative controls. Plants were transferred to growth chambers with temperatures of 35ºC/28ºC (day/night) and 60-65% relative humidity for eight days. Plants were monitored each day and lesions were measured on day eight.

For panicle spray inoculation, overnight cultures of *B. glumae*, *P. fluorescens* and *B. cenocepacia* were diluted to OD_{600}= 1 ($1X10^8$ CFU/mL) in water containing 1% Tween 20. Three to four emerging panicles from 'Wells' were sprayed thoroughly with 20mL of inoculum using an airbrush, either *B. glumae* alone, *B. glumae/ B. cenocepacia* co-inoculation, or *B. glumae/ P.*

fluorescens co-inoculation. Plants were transferred to a growth chamber with temperatures 35ºC/28ºC (day/night) and 60-65% relative humidity. Panicles were then covered in autoclave plastic bags for 48 hours and then left uncovered in the growth chamber for an additional 24 hours. Disease symptoms were evaluated at 3 days post inoculation by counting the number of discolored spikelets and the total number of spikelets per panicle to calculate percentage of discoloration.

Persistence Assay

Overnight cultures of *B. cenocepacia* and *P. fluorescens* were diluted to OD_{600}= 0.125 ($1x10^8$ CFU/mL). Four-week old 'Wells' plants were inoculated with 20µL *B. cenocepacia* or *P. fluorescens* by sheath injection. Plants were transferred to a growth chamber with temperatures 35ºC/28ºC (day/night) and 60-65% relative humidity and grown for 5, 10 and 20 days. At 0, 5, 10 and 20 days post-inoculation, aboveground portions of the plants were harvested, weighed, and ground in a sterile mortar and pestle with 1mL sterile water. Ground tissues were serially diluted and plated on KB plates that were incubated at 30ºC for 24 h or until individual colonies developed.

Results

Isolation of culturable bacterial populations from 'Bengal' and 'Jupiter'

Sequence analysis of the 16S rRNA gene of bacterial strains isolated from seeds revealed that the most abundant culturable bacterial strains in both genotypes are: *Pseudomonas sp*. NF81 that makes up 33.33% of the Bengal seed culturable bacterial population and 37.5% of the Jupiter seed culturable bacterial population (Figure 1). Less abundant strains included *Pseudomonas tolaasi* strain GD76 at 6.25% in Jupiter and 5.56% in Bengal, *Pseudomonas putida* strain PSDM3 at 6.25% in Jupiter and 5.56% in Bengal, and *Pseudomonas putida* strain FB15 at 25% in Jupiter and 11.1% in Bengal. Three Jupiter-specific bacteria were found on the

seeds, *Pseudomonas fulva* strain Pful-2 (6.25%), *Stenotrophomonas sp*. DIV102 (12.5%), and *Pseudomonas gessardii* strain 5611 (6.25%). Bengal-specific strains included *Pantoea sp. 3030* (5.56%)*, Pseudomonas sp. GE-52* (5.56%)*, Stenotrophomonas sp.* (5.56%)*, Stenotrophomonas sp. 2012A* (5.56%)*, Pseudomonas sp. strain DDM8* (5.56%)*, Pseudomonas sp. GAO7 (*11.1%) and *Pseudomonas sp. G0838* (5.56%) in seed (Figure 1).

Comparison of the leaf culturable bacterial population of Bengal and Jupiter showed that the most prevalent strain of bacteria in both Bengal and Jupiter was *Pseudomonas sp*. YSA5, making up 33% of the Jupiter leaf culturable bacterial population and 40% of the Bengal leaf culturable bacterial population. Less abundant strains included *Pseudomonas sp.* GE-52 at 10% in Jupiter and 10% in Bengal, *Bacterium* KLnb3 at 10% in Jupiter and 5% in Bengal, *Pseudomonas pseudoalcaligenes* strain MHF ENV 11 at 5% in Jupiter and 10% in Bengal, and *Pseudomonas sp.* ABC at 5% in Jupiter and 5% in Bengal. Five Jupiter- specific strains were isolated from the leaf culturable bacterial population; *Pseudomonas putida strain MR1* (5%)*, Pseudomonas japonica strain ICE217* (5%)*, Pseudomonas sp. strain S2-3* (5%)*, Pseudomonas sp. NY10-1* (10%) *and Pseudomonas fluorescens strain Bp-15* (5%) (Figure 2). Bengal-specific strains included *Pseudomonas sp.* strain DDM8 (25%) and *Pseudomonas sp.* S28 (5%) (2015) in leaf (Figure 2).

Overall, sequence analysis of the 16S rRNA gene of bacterial strains isolated revealed that the genera *Pseudomonas* is ubiquitous regardless of genotype or tissue. However, it was possible to identify unique genera present in seeds and leaves from the different genotypes. Some genera were present only in the seeds or leaves and some were present only in one of the genotypes.

Validation of *B. glumae* identity

Because the identification of the *B. glumae* strains was only based on their growth in the semi-selective media CCNT (Kawardani et al., 2000), which can lead to mis-identification, the

identity of those strains was confirmed by PCR using the following published primers: Bg 23S-ITS1F(TGCTACGAAGAGCACTCTAAG), R (ACATGCACTTGTTCGCTTG), Bg specific F (ACGTTCAGGGATACTGAGCAG), R (AGTCTGTCTCGCTCTCCCGA)
Bg 23S ITS-2 F (ACACGGAACACCTGGGTA), R (TCGCTCTCCCGAAGAGAT)
and Bg gyrB F (GAAGTGTCGCCGATGGAG), R (CCTTCACCGACAGCACGCAT) (Karki et al., 2012). All the primers successfully generated PCR product when using UAPB10 and UAPB11 as templates. Since Bg 23S ITS-1, ITS-2 and gyrB are conserved in bacteria, the lack of amplification of UAPB12 and 13 with those primers is probably due to poor quality of the templates. A PCR product was obtained with the Bg specific primer in UAPB13 but the equivalent band was very weak in UAPB12. Because the Bg specific is supposed to amplify only *B. glumae,* this primers pair was considered diagnostic for *B. glumae* and the positive amplification indicates that UAPB10, UAPB11 and UAPB13 are all *B. glumae.* Further sequence analysis of PCR products after amplification with the 16S rRNA primers confirmed that UAPB 10, UAPB 11 and UAPB 13 were indeed *B. glumae,* while UAPB 12 is *Klebiella pneumonia*.

Screening of bacterial strains antagonistic activity against *B. glumae*

Five bacterial strains specifically associated with seeds or leaves of the genotype Jupiter were chosen to investigate growth inhibition against *B. glumae* (UAPB13). Those strains are: *Pseudomonas fulva strain Pful-2, Pseudomonas putida strain MR1, Pseudomonas japonica strain ICE217, Pseudomonas sp. Strain S2-3* and *Pseudomonas fluorescens strain Bp-15*. In parallel, 10 strains of bacteria from the Rojas lab collection were also tested. The five strains isolated from Jupiter did not show a strong inhibitory effect against *B. glumae* (Figure 4A). However, out of the 10 laboratory strains, seven: PBL3, PBL9, PBL15, PBL16, PBL18, PBL19 and PBL20 caused a visible zone of growth inhibition (Figure 4A). Calculations on the areas of zone inhibition showed that PBL3, PBL18 and PBL 20 had the largest areas of the zones of

inhibition: 334.7mm^2, 415.6mm^2 and 450.5mm^2, respectively (Figure 4B). Therefore, these strains were chosen for further characterization.

Identification of strains with antagonistic activity against *B. glumae*

To further characterize the inhibitory bacterial strains PBL3, PBL18, and PBL20, genomic DNA was extracted and the 16s rRNA gene was amplified by PCR using 16s rRNA primers. The 16s rRNA sequence of PBL3 showed 100% identity at the nucleotide level to the 16s rRNA gene of *Pseudomonas fluorescens* while the 16s rRNA sequence of PBL18 and PBL20 showed 100% identity at the nucleotide level to the 16s rRNA gene of *Burkholderia cenocepacia* (PBL18). PBL18 was chosen for the remainder of the study.

***B. cenocepacia* and *P. fluorescens* inhibit growth of three *B. glumae* strains**

To determine whether *B. cenocepacia* and *P. fluorescens* inhibited growth for *B. glumae* in general or just *B. glumae* UAPB13, two other strains of *B. glumae,* UAPB10 and UAPB11 were tested in the inhibition experiments. All three strains presented large zones of inhibition when grown with a filter paper disk containing either PBL3 or PBL18 (Figure 5A). When tested with *P. fluorescens,* the zones of inhibition were 318.8mm^2 for UAPB10, 287.4mm^2 for UAPB 11 and 353.4mm^2 for UAPB 13 (Figure 5B). When tested with *B. cenocepacia*, the zones of inhibition were 274.1mm^2 for UAPB 10, for 302.3mm^2 UAPB 11 and 318.8mm^2 for UAPB 13 (Figure 5B). Statistical analysis showed that there was not a statistically significant difference in growth inhibition of the different strains of *B. glumae* by *B. cenocepacia* or *P. fluorescens.* Therefore, UAPB13 was used for the remaining of the experiments because the Rojas lab had already generated a gentamycin resistant derivative of UAPB13, which will be used in subsequent experiments.

***P. fluorescens* and *B. cenocepacia* reduce disease symptoms caused by *B. glumae* by sheath injection.**

To determine if *B. cenocepacia* and *P. fluorescens* could be effective biological control agents against *B. glumae, B. cenocepacia* and *P. fluorescens* were co-inoculated with *B. glumae* by sheath injection (Figure 6). *E.coli* was also co-inoculated with *B. glumae* as control. Plants inoculated with *B. glumae* alone showed disease symptoms in the stem characterized by brown lesions surrounding the area of inoculation (Figure 6A) and had average lesion lengths of 58.3mm (Figure 6B). Similar disease symptoms were obtained when co-inoculating *B. glumae* with *E. coli* and in that case the average lesion lengths was 71.6mm (Figure 6B). However, plants that were inoculated with the combinations of *B. glumae*/*B. cenocepacia* or *B. glumae*/*P. fluorescens* had very small lesions (Figure 6A) that measured 1 mm when plants were inoculated with *B. glumae/B. cenocepacia* and 3.6mm when plants were co-inoculated with *B glumae* and *P. fluorescens* (Figure 6B).

Because in the field, the symptoms of *B. glumae* infection are mainly seen in the panicle, it was necessary to test the effectiveness of *P. fluorescens* and *B. cenocepacia* reducing disease symptoms caused by *B. glumae* in panicles. Thus, panicle co-inoculations were carried out (Figure 7). At three days post inoculation plants inoculated with *B. glumae* alone had an average of 25.26% discolored panicles, while plants co-inoculated with *B. glumae* and *B. cenocepacia* had 13.99% discolored panicles (Figure 7b). These results showed that, *B. cenocepacia* is effective at reducing symptoms of *B. glumae* infection (p value = 0.0109). Unfortunately, results for *P. fluorescens* panicle co-inoculations were inconsistent with an average of 16.51% discolored panicles and a large error bar resulting in a non-significant reduction of symptoms (p value= 0.2431).

B. cenocepacia and *P. fluorescens* do not persist *in planta*

To investigate the feasibility of using *P. fluorescens* and *B. cenocepacia* as true biological control agents against *B. glumae*, the long-term persistence of these strains was evaluated by inoculating them in rice and retrieving after 5, 10 and 20 days. Inoculation of rice with *B. cenocepacia* showed that *B. cenocepacia* growth increases from day 0 to 5, then decreases from day 10 to 20 resulting in an overall decrease in the amount of bacteria found in the plant after twenty days. Although it appears that there is a trend toward increase in bacterial numbers from 0 to 10 dpi, the statistical analysis showed that the numbers of bacteria at those time points are not significantly different. However, the numbers of bacteria at 20 dpi are significantly lower than at 10 dpi, but equivalent to the initial numbers at 0dpi (Figure 8). Inoculation of rice with *P. fluorescens* showed that there are equivalent numbers of bacteria between 0 and 5 days indicating that *P. fluorescens* did not grow. Moreover, after 5 days, there is a progressive decline in bacterial numbers resulting in a significant reduction in bacterial numbers (Figure 8). This data suggests that *B. cenocepacia* and *P. fluorescens* are unable to persist or proliferate in the plant and therefore would make poor biological control agents in the field.

Discussion

This study was a proof-of-concept to evaluate bacterial populations between a rice genotype that is moderately resistant to *B. glumae* (Jupiter) and another that is susceptible (Bengal). The study investigated leaves and seeds as they are relevant to the biology of *B. glumae.* Analysis of the bacterial communities associated with rice seeds have not been done before, but several studies have investigated the bacterial communities associated with rice leaves and roots. In such studies, the bacterial community in the leaves was found to be different and less complex than that of the rhizosphere, but comparable to the bacterial communities in the phyllosphere of other plant species (Knief et al.,2012).

Analyses of the bacterial communities in the leaves of several plants have shown that they are represented by few bacterial genera, the most prevalent being *Pseudomonas, Sphingomonas*, *Methylobacterium*, *Bacillus*, *Massilia*, *Arthrobacter* and *Pantoea* (Bulgarelli et al.,2013). In this study, the most common bacterial genera associated with rice seeds and leaves was *Pseudomonas spp.*, which is not surprising considering the versatility of this bacteria colonizing different environments (Piex et al., 2009). Specifically, *Pseudomonas sp. NF81* was the most prevalent isolate in seeds and represented 33.33% of the culturable bacteria in Bengal and 37.5% of the culturable bacteria in Jupiter. *Pseudomonas sp. YSA5* was the most prevalent isolate in leaves and represented 33% of the culturable bacteria in Bengal and 40% of the culturable bacteria in Jupiter. While *Pseudomonas* sp. was present in both Jupiter and Bengal, its frequency and diversity was larger in Jupiter. The leaves in Bengal also have a high percentage of *Pseudomonas* sp. strain DDM8 which is not present in the leaves of genotype Jupiter.

When comparing the microbial diversity between Jupiter and Bengal, the results showed that the bacterial composition is different from each other in both leaves and seeds, although those differences do not appear to be too dramatic. The genotype-mediated differences in the composition of the microbial communities associated with plants has been observed before (Wagner et al., 2016). Although it is still not fully understood how specific plant genotypes recruit specific microorganisms, a model for the recruitment of microorganisms to the roots has been proposed. In this model microorganisms in the soil are recruited to the root and, at later stages the genotype of the plant fine-tunes the final microbial composition to fit the plant's unique environment and needs (Bulgarelli et al., 2013). It is possible that similar mechanisms operate in leaves and accounts for the differences between Jupiter and Bengal.

Whereas there were specific genera associated with each genotype in each organ, common bacterial genera were retrieved within each organ. In other words, most of the differences were found between organs than between genotypes. For example, *Pseudomonas*

NF81 was retrieved multiple times from seeds in both genotypes but, it was not found at all in leaves. *Pseudomonas sp.* strain DDM8 was plentiful and unique only to Bengal leaves and was not found on seeds at all. *Pseudomonas YSA5* was abundant in leaves from both genotypes but was not found in seeds. The similarities between the culturable bacterial population of organ types (leaves and seeds) being stronger than genotype (Bengal and Jupiter) shows a stronger relationship between culturable bacterial population and tissue environment than host genotype. Similar findings were found with studies evaluating microbial populations in roots (Lundberg et al., 2012).

Although this study was not comprehensive, it is promising for future high-throughput studies aimed at unraveling the genetic basis of microbial recruitment in plants. The finding that indeed the microbial composition between two rice genotypes differs suggests that those differences could account for varying degrees of resistance and susceptibility to *B. glumae* as has been suggested before for soil microbes and their effect on root health (Mendes et al., 2011). Knowledge of the microbial composition of crops is important as, in the future, scientists may be able to manipulate the microbial composition of crops to influence plant health and other characteristics (Busby et al., 2017).

Considering that unique bacteria were found in Jupiter: *Pseudomonas fulva* strain Pful-2, *Pseudomonas putida* strain MR1, *Pseudomonas japonica* strain ICE217, *Pseudomonas* sp. strain S2-3 and *Pseudomonas fluorescens* strain Bp-15, this work sought to investigate if those unique bacteria had antagonistic activity against *B. glumae.* The first step towards determining whether a bacterial strain or a particular compound has antagonistic activity is to conduct growth inhibition test *in vitro.* The inhibition assay used in this chapter was an improvement from previous work investigating rice associated bacteria (Shresta et al.,2016), because it provided even distribution of *B. glumae* on the agar to reproducibly assess the potential growth inhibition. Although the five strains that were unique to Jupiter did not show growth inhibition of *B. glumae,*

three strains from the laboratory collection showed large zones of growth inhibition and those strains were identified as *P. fluorescens* and *B. cenocepacia* and those strains were further used for *in planta* experiments.

In contrast to previous work where rice-associated bacteria showing growth inhibition in vitro did not have reproducible effects when inoculated in plants (Shresta et al. 2016), in this work, co-inoculation of *P. fluorescens* and *B. cenocepacia* with *B. glumae* consistently reduced disease symptoms. This effect can be directly attributed to these bacterial strains as plants inoculated with the combination *B. glumae*/*E. coli* still had disease symptoms that resemble the symptoms observed by *B. glumae* alone.

The finding that *P. fluorescens* had antagonistic activities is not surprising as *Pseudomonas* spp. are commonly found in suppressive soils and have been well-studied and used as biological control agents against soil-borne pathogens in the past (Weller, 2007; Kyselkova et al., 2009). Another strain of *Pseudomonas, Pseudomonas sp.* Rh323 was effective controlling *Xanthomonas oryzae* pv. *oryzae* and, reduced disease symptoms associated with the disease under field conditions (Yasmin et al., 2016). Similar to *P. fluorescens, Burkholderia spp* have also been identified as biological control agents for plant diseases including *Fusarium moniliforme* on maize (Hebbar et al., 1992), *Pythium* Damping-off (Bowers and Parke, 1993) and *Rhizoctonia* stem rot (Cartwright and Benson, 1995).

While both *P. fluorescens* and *B. cenocepacia* were effective in reducing disease under controlled conditions, more work is needed to investigate conditions to increase its persistence. Neither one of them showed good persistence in leaves after sheath injection, but as soil-borne bacteria, it is possible that they have higher persistence in the soil, the preferred habitat for these bacteria. If that is the case, the antagonistic activities of these bacteria will be relevant to control bacterial panicle blight at early stages of the disease when the bacteria is associated with the seed.

The use of *B. cenocepacia* as a biological control agent is unlikely as this bacterium is a member of the *Burkholderia cepacia* complex (Bcc) , a group of opportunistic pathogens of humans and are especially dangerous to individuals with cystic fibrosis (Parke and Gurian-Sherman, 2001). Therefore, its use could pose a risk to human health. However, the isolation of compounds produced by these bacteria will pave the way towards controlling bacterial panicle blight of rice.

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Appendix

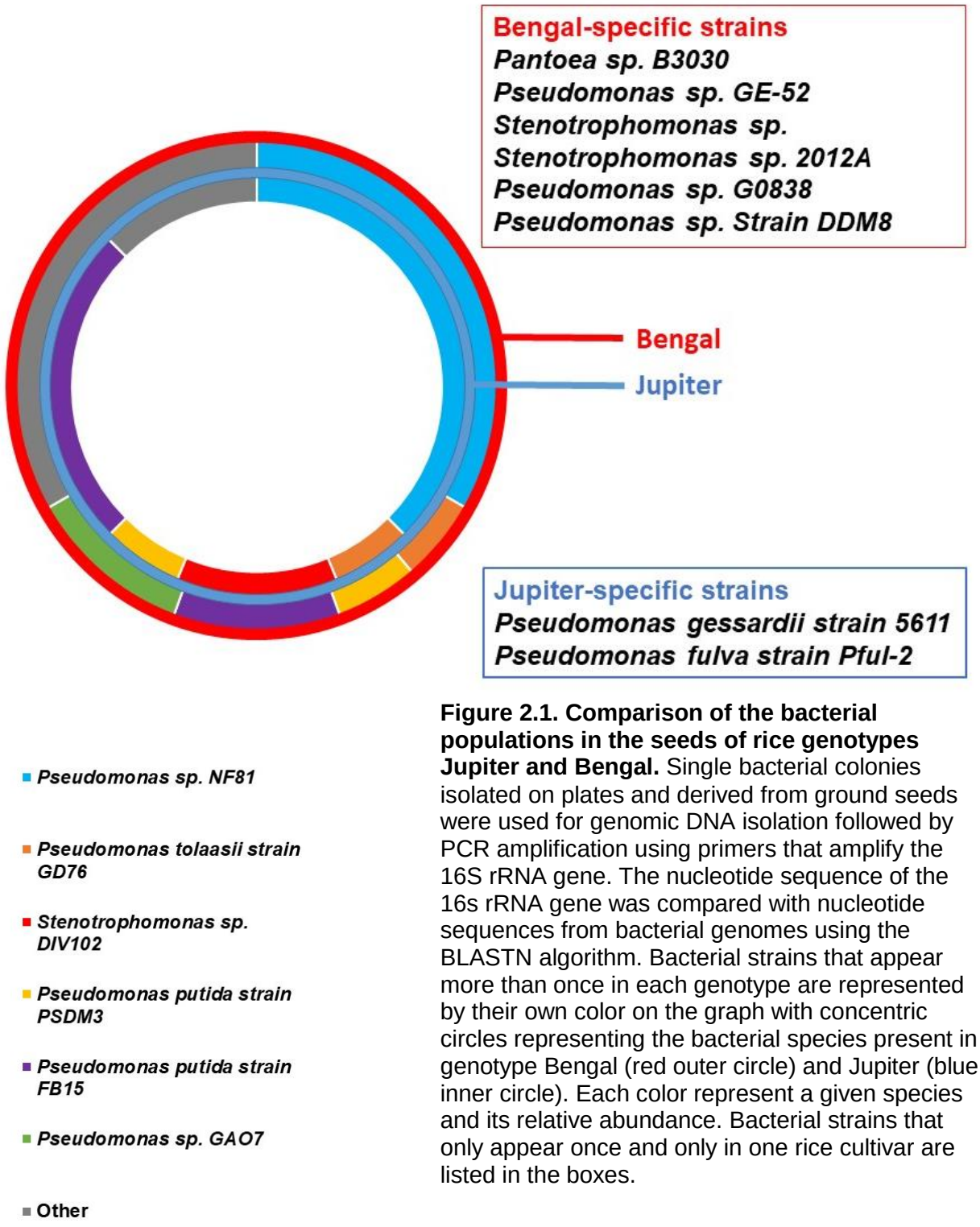


Figure 2.1. Comparison of the bacterial populations in the seeds of rice genotypes Jupiter and Bengal. Single bacterial colonies isolated on plates and derived from ground seeds were used for genomic DNA isolation followed by PCR amplification using primers that amplify the 16S rRNA gene. The nucleotide sequence of the 16s rRNA gene was compared with nucleotide sequences from bacterial genomes using the BLASTN algorithm. Bacterial strains that appear more than once in each genotype are represented by their own color on the graph with concentric circles representing the bacterial species present in genotype Bengal (red outer circle) and Jupiter (blue inner circle). Each color represent a given species and its relative abundance. Bacterial strains that only appear once and only in one rice cultivar are listed in the boxes.

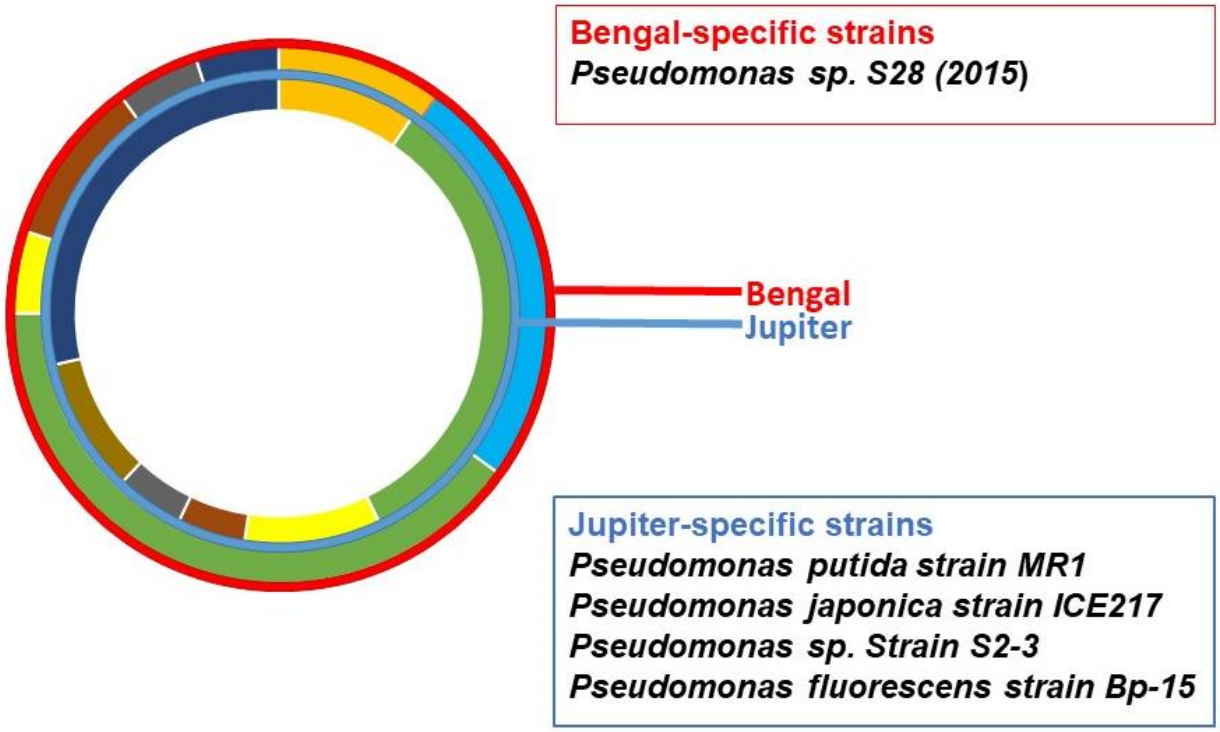


Figure 2.2. Comparison of the leaf microbiome in rice genotypes Jupiter and Bengal. Single bacterial colonies isolated on plates and derived from ground seeds were used for genomic DNA isolation followed by PCR amplification using primers that amplify the 16S rRNA gene. The nucleotide sequence of the 16s rRNA gene was compared with nucleotide sequences from bacterial genomes using the BLASTN algorithm. Bacterial strains that appear more than once in each genotype are represented by their own color on the graph with concentric circles representing the bacterial species present in genotype Bengal (red outer circle) and Jupiter (blue inner circle). Each color represent a given species and its relative abundance. Bacterial strains that only appear once and only in one rice cultivar are listed in the boxes.

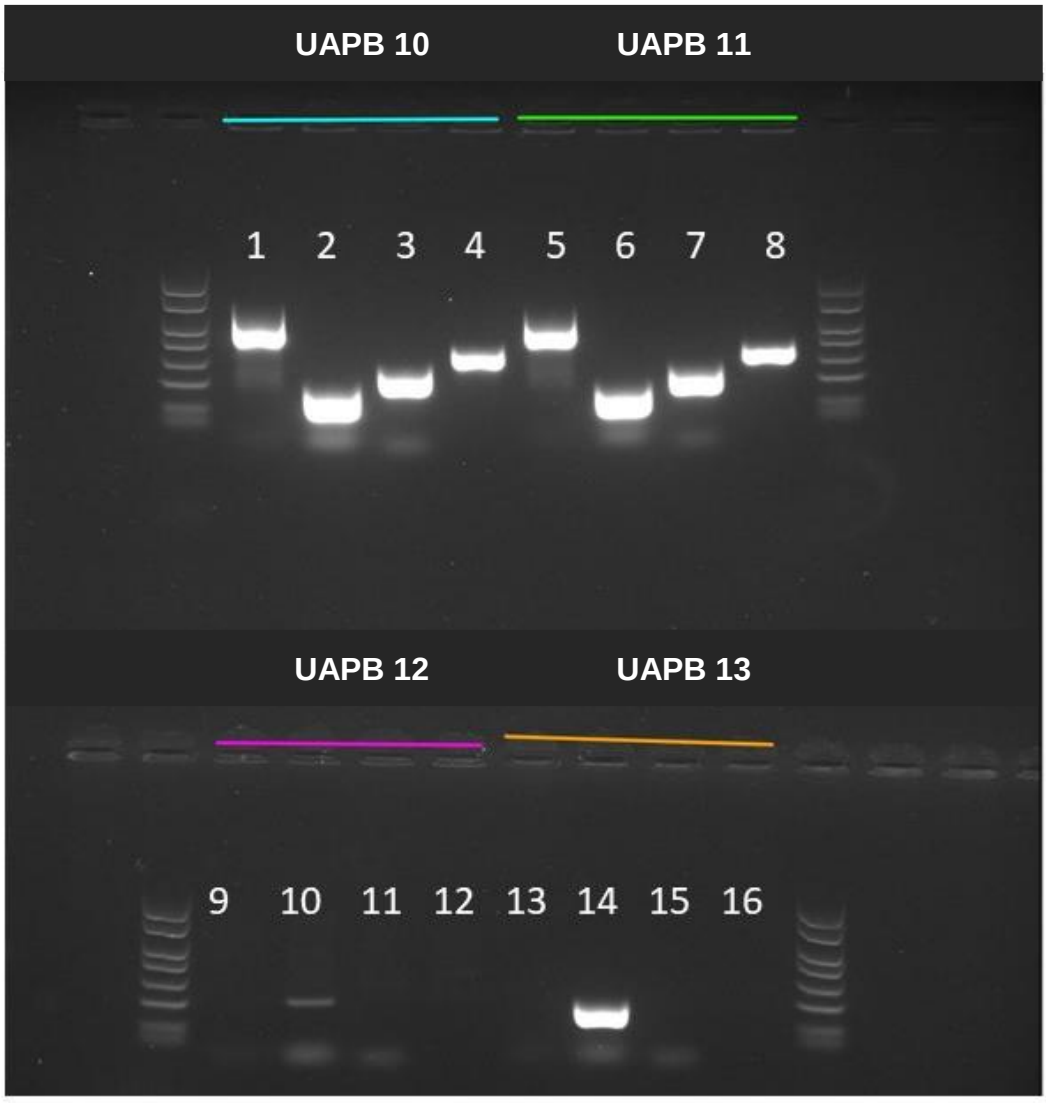


Figure 2.3. Validation of the identity of *B. glumae.* Genomic DNA was isolated from strains UAPB10, UAPB11, UAPB12 and UAPB13 and used as template for PCR reaction using primers: Bg 23S ITS (lanes 1, 5, 9 and 13), Bg specific (lanes 2, 6, 10 and 14), Bg 23S ITS-2 (lanes 3, 7, 11, 15) and Bg gyrB (lanes 4, 8, 12, 16).

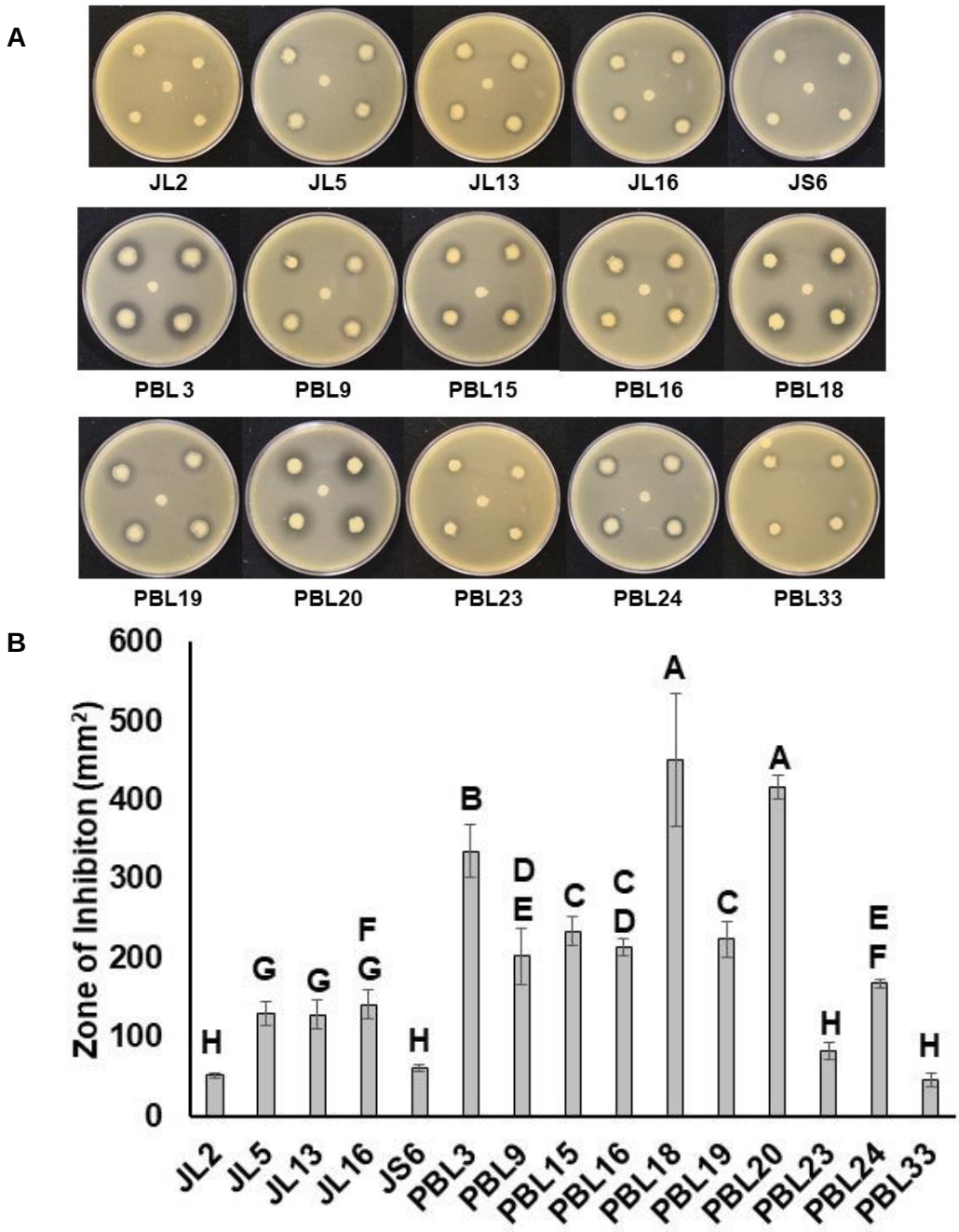


Figure 2.4. Identification of bacteria with antagonistic activities against *B. glumae.* Five bacterial strains isolated from leaves and seeds from rice cultivar Jupiter (JL2, JL5 JL13, JL16, JS6) and 10 bacterial strains from the Rojas Lab collection (PBL3, PBL9, PBL15, PBL16, PBL18, PBL19, PBL20, PBL23, PBL24, PBL33) were tested for their ability to inhibit growth of *B. glumae* (UAPB13). *B. glumae* at OD600 =0.001 were mixed with molten KB agar and five sterile filter paper disks were placed on the agar surface. Five microliters of bacterial strains at OD600= 1 were pipetted onto four of the filter paper disk. Water was added to the fifth disk (in center) to be used as control. Plates were incubated at 30^{o}C for 48h. Clear zones of growth inhibition in (A) were measured (B). The differences among means was calculated using ANOVA statistical analysis with a p value <0.0001.

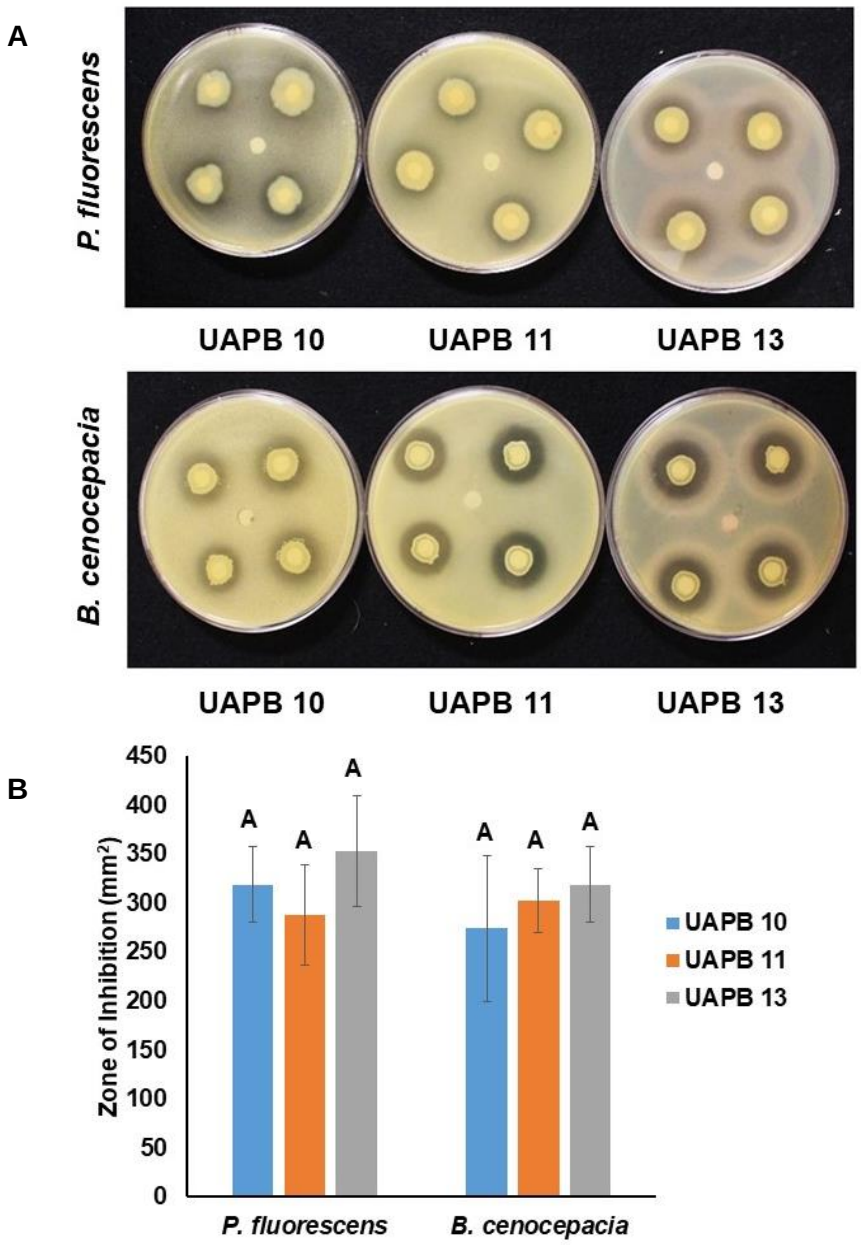
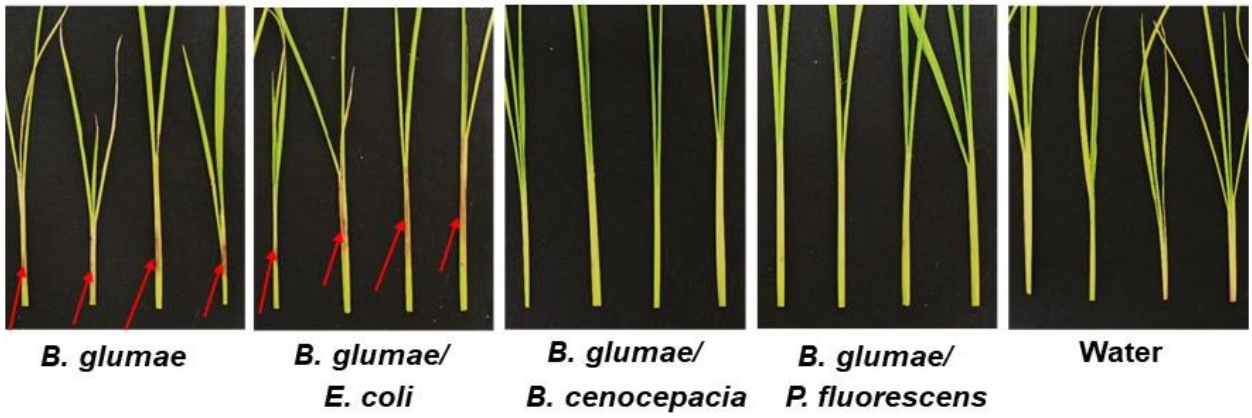


Figure 2.5. *P. fluorescens* and *B. cenocepacia* cause growth inhibition of *B. glumae* strains UAPB 10, 11, 13. *P. fluorescens* and *B. cenocepacia* were tested for their ability to inhibit the growth of three strains of *B. glumae* (UAPB10, UAPB11 and UAPB13). *B. glumae* at OD600= 0.01 were mixed with molten KB and *B. glumae* and five filter paper disks were placed on the agar surface. Five microliters of bacterial strains at OD600= 1 were pipetted onto four of the filter paper disks with a water control disk in the center and plates were incubated at 30^{o}C for 48h. Water was pipetted onto the center disc as a negative control. Clear zones of growth inhibition in (A) were measured and graphed is shown in (B). Comparisons among the average zone of growth inhibition for UAPB10, UAPB11 and UAPB13 when exposed to either *P. fluorescens* or *B. cenocepacia* was analyzed using ANOVA with P values= 0.7180 and 0.3474, respectively.

A



B

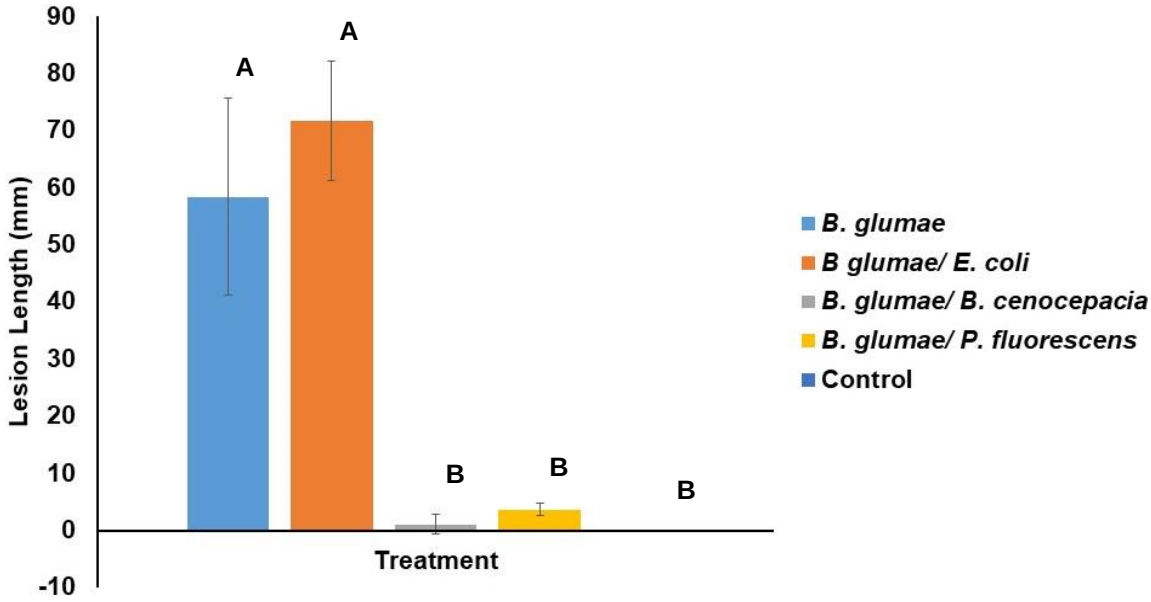


Figure 2.6. Co-inoculation of *B. glumae* with *B. cenocepacia* or *P. fluorescens* reduce disease symptoms in rice. Eight-week-old rice plants from cultivar Wells were inoculated in the sheath with *B. glumae* alone or in combination with *E. coli, B. cenocepacia* or *P. fluorescens,* or mock-treated with water. Disease symptoms were evaluated after eight days (A). Bars represent average lesion lengths for three replicates (B). Treatments were compared using ANOVA statistical analysis with a P- value <0.001.

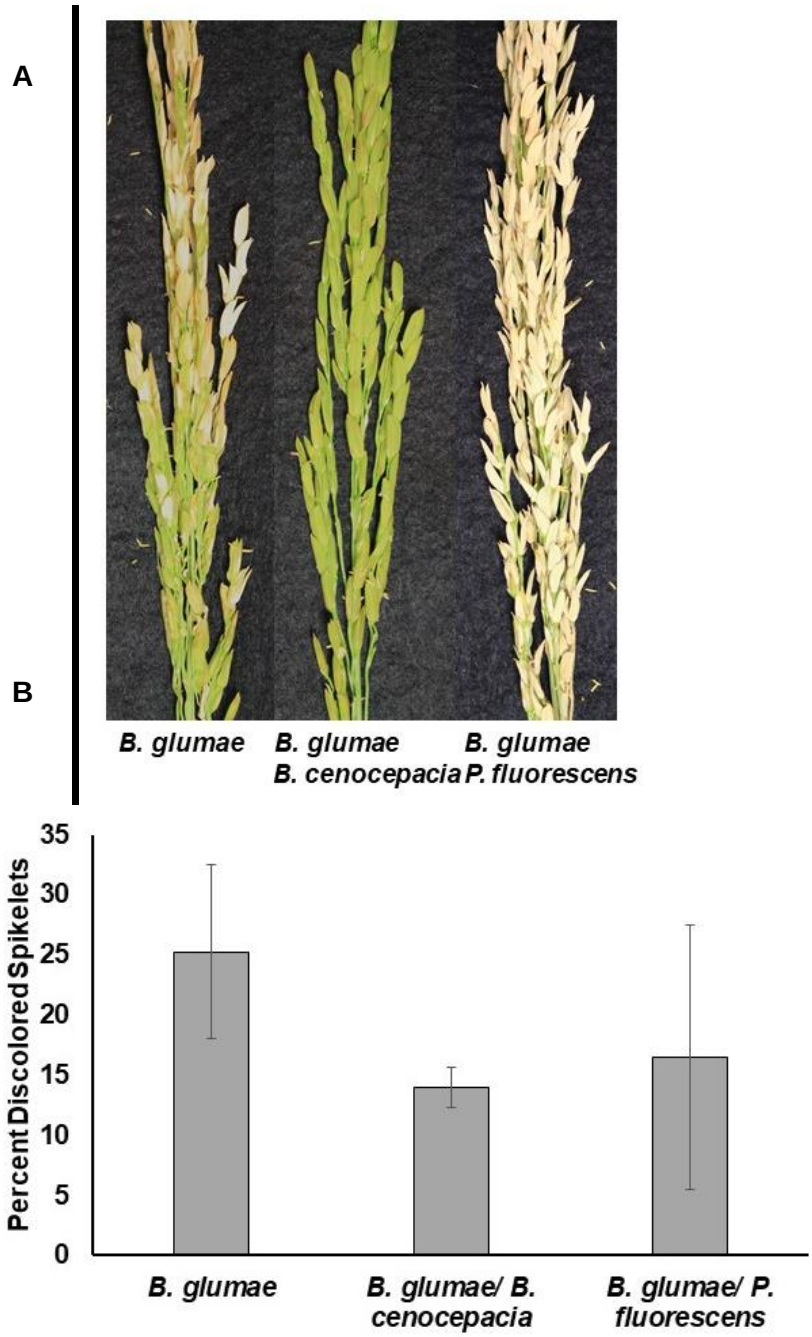


Figure 2.7. Co-inoculation of *B. glumae* with *B. cenocepacia* reduces disease symptoms on rice panicles. Emerging panicles from cultivar Wells were sprayed with 20mL of either *B. glumae* inoculum OD_{600}= 1 or a co-inoculatant of *B. glumae* OD_{600}= 1 and *B. cenocepacia* OD_{600}= 1 or a co-inoculation of B. glumae OD_{600} =1 and *P. fluorescens* OD_{600}=1. Symptoms were observed after 3 dpi (A) and number of discolored spikelets counted. Bars in (B) represent the percent of discolored spikelets. Data was analyzed using a t test with a p value = 0.0109 for *B. glumae* vs *B. glumae/ B. cenocepacia* and p- value = 0.2431 for *B. glumae* vs *B.glumae/P.fluorescens*.

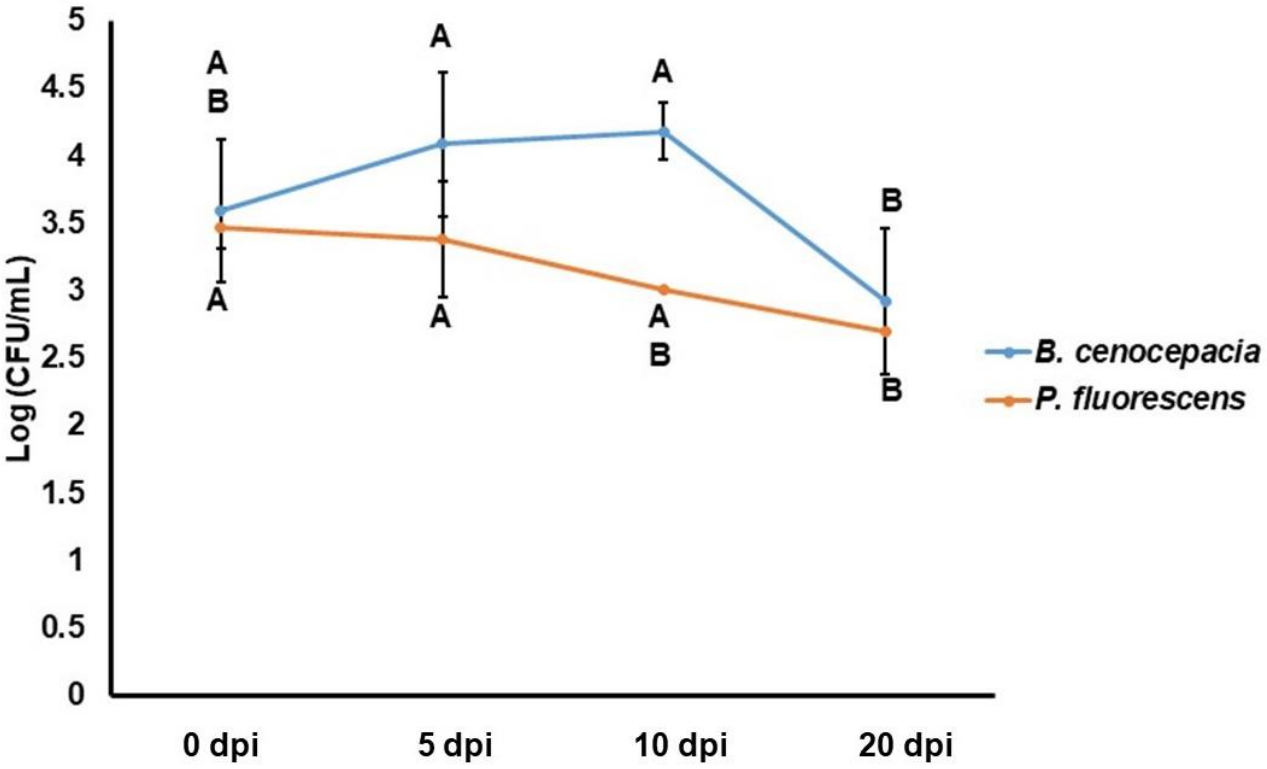


Figure 2.8. *B. cenocepacia* and *P. fluorescens* do not have long-term persistence in rice. Four-week-old rice plants cultivar Wells were inoculated with 20µL OD_{600}=0.125 *B. cenocepacia* or *P. fluorescens*. Plants were ground and diluted on KB plates at 0, 5, 10 and 20 days to determine persistence of the bacteria in plant tissues. The comparison of bacterial numbers over time for *B. cenocepacia* and *P. fluorescens* was analyzed using ANOVA. P-value= 0.1257 for *B. cenocepacia* and 0.0589 for *P. fluorescens*.

Table 1. Bacteria isolated from seeds of cultivars Jupiter and Bengal

Isolate number	Closest species in GenBank based on 16S rDNA sequence
BS1	*Pseudomonas sp. NF81*
BS2	*Pseudomonas sp. NF81*
BS3	*Pseudomonas tolaasii strain GD76*
BS4	*Pseudomonas sp. NF81*
BS5	*Pantoea sp. B3030*
BS6	*Pseudomonas sp. NF81*
BS7	*Pseudomonas sp. GE-52*
BS8	*Pseudomonas sp. NF81*
BS9	*Stenotrophomonas sp.*
BS10	*Pseudomonas sp. NF81*
BS11	*N/A*
BS12	*Pseudomonas putida strain FB15*
BS13	*Pseudomonas sp. GAO7*
BS14	*Stenotrophomonas sp. 2012A*
BS15	*Pseudomonas putida strain PSDM3*
BS16	Poor quality sequence
BS17	*Pseudomonas sp. GAO7*
BS18	*Pseudomonas putida strain FB15*
BS19	*Pseudomonas sp. G0838*
BS20	*Pseudomonas sp. Strain DDM8*
JS1	*Pseudomonas sp. NF81*
JS2	*Pseudomonas sp. NF81*
JS3	*Pseudomonas sp. NF81*
JS4	*Pseudomonas tolaasii strain GD76*
JS5	*Pseudomonas sp. NF81*
JS6	*Pseudomonas fulva strain Pful-2*
JS7	*Stenotrophomonas sp. DIV102*
JS8	*Pseudomonas sp. NF81*
JS9	*Pseudomonas sp. NF81*
JS10	*Stenotrophomonas sp. DIV102*
JS11	*Pseudomonas putida strain FB15*
JS12	*Pseudomonas gessardii strain 5611*
JS13	Poor quality sequence
JS14	*Pseudomonas putida strain FB15*
JS15	Poor quality sequence
JS16	*Pseudomonas putida strain FB15*
JS17	Poor quality sequence
JS18	Poor quality sequence
JS19	*Pseudomonas putida strain PSDM3*
JS20	*Pseudomonas putida strain FB15*

Table 2. Bacteria isolated from leaves of cultivars Jupiter and Bengal

Isolate number	Closest species in GenBank based on 16 s rDNA sequence
BL1	*Pseudomonas sp. strain DDM8*
BL2	*Pseudomonas sp. GE-52*
BL3	*Pseudomonas pseudoalcaligenes strain MHF ENV 11*
BL4	*Pseudomonas sp. YSA5*
BL5	*Bacterium KLnb3 / Pseudomonas sp. strain AC530*
BL6	*Pseudomonas pseudoalcaligenes strain MHF ENV 11*
BL7	*Pseudomonas sp. Strain DDM8*
BL8	*Pseudomonas sp. S28 (2015)*
BL9	*Pseudomonas sp. Strain DDM8*
BL10	*Pseudomonas sp. YSA5*
BL11	*Pseudomonas sp. Strain DDM8*
BL12	*Pseudomonas sp. YSA5*
BL13	*Pseudomonas sp. YSA5*
BL14	*Pseudomonas sp. YSA5*
BL15	*Pseudomonas sp. GE-52*
BL16	*Pseudomonas sp. ACB*
BL17	*Pseudomonas sp. YSA5*
BL18	*Pseudomonas sp. Strain DDM8*
BL19	*Pseudomonas sp. YSA5*
BL20	*Pseudomonas sp. YSA5*
JL1	*Pseudomonas sp. NY10-1*
JL2	*Bacterium KLnb3 / Pseudomonas fluorescens strain Bp-15*
JL3	*Pseudomonas sp. YSA5*
JL4	*Pseudomonas sp. YSA5*
JL5	*Pseudomonas sp. Strain S2-3*
JL6	*Pseudomonas sp. YSA5*
JL7	*Pseudomonas sp. GE-52*
JL8	Poor quality sequence
JL9	*Pseudomonas pseudoalcaligenes strain MHF ENV 11*
JL10	*Pseudomonas sp. YSA5*
JL11	*Bacterium KLnb3 / Pseudomonas sp. Strain AC530*
JL12	*Bacterium KLnb3 / Pseudomonas sp. Strain AC530*
JL13	*Pseudomonas japonica strain ICE217*
JL14	*Pseudomonas sp. ACB*
JL15	*Pseudomonas sp. YSA5*
JL16	*Pseudomonas putida strain MR1*
JL17	*Pseudomonas sp. YSA5*
JL18	*Pseudomonas sp. YSA5*
JL19	*Pseudomonas sp. NY10-1*
JL20	*Pseudomonas sp. GE-52*

Table 3. Bacterial strains used in this study

ID	Strain	Source
UAPB10	*Burkholderia glumae*	Dr. Yeshi Wamishe
UAPB11	*Burkholderia glumae*	Dr. Yeshi Wamishe
UAPB13	*Burkholderia glumae*	Dr. Yeshi Wamishe
PBL9	*P. fluorescens 5-40*	This work
PBL3	*P. fluorescens PFS JA 4092*	This work
PBL16	*1-30 10-99 P. fluorescens*	This work
PBL20	*B. cenocepacia*	This work
PBL33	*Burkholderia sp.*	This work
PBL19	*5-40 10-1999 P. fluorescens*	This work
PBL15	*1-30 10-99 P. fluorescens*	This work
PBL24	*P. fluorescens 2-79*	This work
PBL18	*B. cenocepacia*	This work
PBL23	*Pseudomonas sp.*	This work
JL2	*Pseudomonas fluorescens strain Bp-15*	This work
JL5	*Pseudomonas sp. Strain S2-3*	This work
JL13	*Pseudomonas japonica strain ICE217*	This work
JL16	*Pseudomonas putida strain MR1*	This work
JS6	*Pseudomonas fulva strain Pful-2*	This work

Chapter 3: Harnessing *Burkholderia cenocepacia* and *Pseudomonas fluorescens* as sources of antimicrobials against *Burkholderia glumae*

Introduction

Microorganisms associated with plants in the rhizosphere or phyllosphere can have antagonistic activities against pathogens, and consequently can be used as biological control agents. Two main mechanisms of biological control are: competition and antibiosis. Through competition a given biological control agent withdraws essential nutrients for pathogen growth, or occupies a specific area that prevent the pathogen from gaining access to the host (Pal and McSpadden Gardener, 2006). Through antibiosis, the biological control agent produces antibiotics, lytic enzymes or waste products that disrupt or kill the pathogen (Pal and McSpadden Gardener, 2006). One example of antibiosis include the control of *Pythium ultimum* by *Trichoderma harzianum*, a fungus that produces enzymes, which damage the cell walls of the pathogen (Benhamou and Chet, 1997). Another classic example of biological control through antibiosis is the control of *Agrobacterium tumefaciens*, by a nonpathogenic bacterium, *Agrobacterium radiobacter*, which produces the antibiotic Agrocin 84 (Kerr, 1980). Although biological control approaches are desirable over chemical control of plant diseases when making ecological considerations, identification of biological control agents is not trivial. However, with the increasing knowledge on the microbial communities associated with plants, it is becoming possible to identify individual microorganisms with biological control properties, or to manipulate those microbial communities to manage diseases and increase plant health (Berg et al., 2009; Busby et al., 2017).

In previous Chapter 2, two bacterial strains with antagonistic activity against *B. glumae* were identified. These antagonistic bacteria are two strains are *B. cenocepacia* and *P. fluorescens,* and their antagonistic activity was demonstrated *in vitro*, with the reduction of growth of *B. glumae*, as well as *in planta,* by reducing disease symptoms caused by *B. glumae,* when they

were co-inoculated with the pathogen. In spite of this promising results, these antagonistic bacteria cannot be used as living biological control agents for the following reasons: 1. *P. fluorescens* and *B. cenocepacia* did not show long-term persistence in rice and, 2. *B. cenocepacia* is an opportunistic human pathogen and therefore their widespread use can have an inherent risk to human health. Notwithstanding these challenges, the finding that these bacteria have antagonistic activity against *B. glumae* makes them useful, initially for research purposes to investigate the mechanisms behind this growth inhibition, and in the future, to isolate molecules responsible for this activity. Thus, this research is significant to advance the development of effective methods to control bacterial panicle blight of rice. This chapter will investigate the mechanisms behind growth inhibition of *B. glumae* by *P. fluorescens* and *B. cenocepacia* and will also identify methods to isolate fractions of *P. fluorescens* and *B. cenocepacia* with antagonistic activity against *B. glumae* in the absence of bacteria.

Methods

Bacterial strains used in this study

B. glumae strain UAPB 13 was collected from rice fields in Stuttgart, AR by Dr. Yeshi Wamishe. *B. glumae* (*pGFP-TIR*) is a derivative of strain UAPB13, which was transformed by electroporation with the plasmid (*pGFP-TIR*) (Miller and Lindow, 1997) that carries GFP and confers gentamycin resistance.

Evaluation of bacterial growth *in vitro*

B. glumae, P. fluorescens, *B. cenocepacia* and *E. coli* were grown on King's B (KB) media plates for two days. Single colonies were transferred to 5 ml KB broth and grown overnight at 30^{o}C with shaking. Overnight cultures were centrifuged at 6,000 rpm for 10 minutes and washed with sterile water three times. Bacterial concentrations were read using a spectrophotometer at OD_{600nm} and diluted to OD_{600}= 0.2 (1x10^{8}CFU/ml) in 100mL KB broth.

Cultures were incubated at 30°C with constant agitation for eight hours. Every 4h, 1mL aliquots were withdrawn to assess bacterial growth by measuring absorbance at OD_{600nm}. This experiment was repeated three times.

Competition assay *in vitro*

A single colony of *B. glumae* (*pGFP-TIR*) was grown in 5ml KB broth at 30°C for 18h with continuous agitation. On the second day 100 µl of this culture were transferred to 100mL of KB broth and grown for 18-24h until it reached an OD_{600}=0.1. On the second day, a single colony of *P. fluorescens* was grown in 5ml of KB broth at 30°C for 18h with continuous agitation and transferred to 100ml of KB broth and grown until reaching at OD_{600}= 0.01. After both strains reached the desired OD_{600}, they were mixed in a 10:1 (*B. glumae: P. fluorescens*) ratio in a total volume of 50 ml. In a different flask, *B. glumae* (*pGFP-TIR*) alone was grown as control. One hundred microliters aliquots were taken at 0, 4, 8, 24 and 48h and these aliquots were serially diluted and plated on KB plates containing gentamycin to enumerate *B. glumae* (*pGFP-TIR*).

Competition Assay *in planta*

Four week-old rice plants cultivar Wells were inoculated by sheath injection with *B. glumae* (*pGFP-TIR*) or with inoculum mixtures *B. glumae* (*pGFP-TIR*)*/ P. fluorescens*. Colonies of *B. glumae* (*pGFP-TIR*) and *P. fluorescens* were grown overnight in liquid LB broth cultures. Cultures were then diluted to OD_{600}= 0.125 in 10mL tubes. Plants were stem injected with 20µL of *B. glumae* (*pGFP-TIR*) or co-inoculated with *B. glumae* (*pGFP-TIR*) OD_{600} = 0.125 and *P. fluorescens* OD_{600} = 0.125. Aboveground portions of inoculated plants were harvested, weighed, ground, serially diluted and plated on gentamycin plates at 0, 3 and 5dpi to count *B. glumae* (*pGFP-TIR*) growth alone and in the presence of *P. fluorescens.* This experiment was repeated three times.

Calculation of competitive Index *in planta*

From the competition assay, bacteria were plated on KB without antibiotics and KB containing gentamycin. *B. glumae* (*pGFP-TIR*) was enumerated by counting bacterial colonies on KB+gentamycin plates. *P. fluorescens* was enumerated by counting bacterial colonies on KB and subtracting from those the number of colonies found on KB+gentamycin plates. The competitive index was calculated as:

$$CI=\frac{\text{cfu } \textit{B. glumae} \text{ GFP/cfu } \textit{P. fluorescens} \text{ (Output)}}{\text{cfu } \textit{B. glumae} \text{ GFP/cfu } \textit{P. fluorescens} \text{ (Input)}}$$ (Macho et al., 2007).

Where output corresponds to the number of both *B. glumae* (*pGFP-TIR*) and *P. fluorescens* counted at 0, 3 and 5 dpi, while input corresponds to the number of bacteria that were inoculated at 0 dpi.

Isolation of cell-free secreted fractions with biological activity from *P. fluorescens* and *B. cenocepacia* obtained from zones of growth inhibition

B. glumae at OD_{600}= 0.01 was mixed with molten agar and 5µl of *P. fluorescens* or *B. cenocepacia* at OD_{600}= 1 were placed on filter paper disks placed on the surface of the agar and incubated for two days to generate the zone of growth inhibition. The portions of the agar containing the zones of inhibition were used to isolate secreted fractions. For that purpose, 3 agar plugs (5mm each) were cut out from the zone of inhibition, transferred to 3 ml of sterile water or KB and incubated at 30^{o}C with constant agitation for 18h. After incubation, water and KB were filter sterilized using a 0.22 µM filter.

To evaluate the biological activity of the cell-free preparations, these preparations were mixed with KB in a 1:1 ratio and inoculated with a single colony of *B. glumae.* KB alone was also inoculated with a single colony of *B. glumae* as control. *B. glumae* either in KB or in KB mixed with cell-free preparations derived from *P. fluorescens* or *B. cenocepacia* were grown for 18h at 30^{o}C and bacterial cultures were serially diluted and plated to enumerate bacteria.

Isolation of cell-free secreted fractions with biological activity obtained from liquid cultures of *P. fluorescens* and *B. cenocepacia*

P. fluorescens and *B. cenocepacia* cultures were grown in 100mL Luria Bertani (LB) broth overnight at 30^{o}C by shaking. Cultures were centrifuged for 10 minutes at 6,000 rpm and the supernatant was collected. Twenty mL of the supernatants were aliquoted into 50 mL flasks and the tops were covered with kimwipes secured with tape to allow for airflow. The samples were then placed in the lyophilizer and dried for 24 hours to remove all water. Pure LB broth samples were lyophilized to be used as control. Lyophilized LB or supernatants from *P. fluorescens* or *B. cenocepacia* cultures were resuspended in sterile water at 0.01 g/ml. Reconstituted LB or bacterial supernatants were filter sterilized using a 0.22 µM filter. To evaluate the biological activity of cell-free preparations, reconstituted fractions were added to KB broth at 1:1 (1.5mL: 1.5mL) ratio and used to grow a single colony of *B. glumae.* As control, a single colony of *B. glumae* was grown on KB. Cultures of *B. glumae* ware grown at 30^{o}C shaker for 24 hours. After 24h, cultures were serially diluted and plated on KB plates to enumerate bacterial populations.

Inoculation of rice plants with *B. glumae* containing lyophilized supernatants from *P. fluorescens* and *B. cenocepacia.*

Lyophilized supernatants from *B. cenocepacia*, *P. fluorescens* or lyophilized LB broth were added to prepare inoculums of *B. glumae* at a concentration of 0.5g/mL. *B. glumae* at OD_{600}=0.125 without added lyophilized materials was used as control. Twenty microliters of these inoculums were injected in triplicates, into the sheath of eight-week old plants from cultivar Wells using insulin syringes. Plants were transferred to growth chamber under with temperatures 35^{o}C/28^{o}C (day/night) and 60-65% relative humidity for eight days. Plants were monitored each day and lesions were measured and photograph after eight days of inoculation.

Results

B. cenocepacia and *P. fluorescens* have a higher growth rate than *B. glumae*

One mechanism of biological control is competition, and since competition involves differential use of nutritional resources between strains one way to start assessing competition is to evaluate the growth rate of bacterial strains in the absence of a competitor. To evaluate the growth rate of *B. cenocepacia, P. fluorescens* and *B. glumae,* these three strains were individually grown starting at early exponential phase and their growth monitored for 8h. As shown in Figure 1, as early as 4h, it was evident that *B. cenocepacia* and *P. fluorescens* were growing faster than *B. glumae* at 30^{o}C and after 8h, they reached OD_{600}= 2.53, and 2.26, respectively while the OD_{600} for *B. glumae* was 1.05 (Figure 1).This data suggests that the faster growth rate of *B. cenocepacia* and *P. fluorescens* in comparison with that of *B. glumae* could enable *B. cenocepacia* and *P. fluorescens* to out-compete *B. glumae* for space and resources as a mechanism for biological control. These results indicate that competition could be a possible mechanism of biological control, in addition to the antibiosis effect that was previously observed.

Competition assay between *B. glumae* and *P. fluorescens in vitro*

To directly evaluate whether *B. cenocepacia* and *P. fluorescens* out-compete *B. glumae,* a direct competition assay was designed wherein the antagonistic bacteria was grown together with *B. glumae* and their growth was evaluated over time. That assay required a way to differentiate both bacteria. Therefore, a gentamycin resistant version of *B. glumae* was used. This assay was only feasible with *P. fluorescens* as this strain is sensitive to gentamycin whereas *B. cenocepacia* is resistant and the antibiotic resistance profiles between *B. cenocepacia* and *B. glumae* was found to be identical (data not shown). As shown in Figure 2, the growth of *B. glumae* (*pGFP-TIR*) was not significantly affected by *P. fluorescens in vitro*. At

each time point, the number of *B. glumae* (*pGFP-TIR*) recovered were equivalent to the numbers of *B. glumae* (*pGFP-TIR*) recovered when grown together with *P. fluorescens*.

Competition assay between *B. glumae* and *P. fluorescens in planta*

Previous results (Chapter 2) showed that co-inoculation of *B. glumae* with *P. fluorescens* reduced disease symptoms in rice and one hypothesis is that *P. fluorescens* competes with *B. glumae*. To test that hypothesis, rice plants were co-inoculated with *B. glumae* (*pGFP-TIR*) and *P. fluorescens* to determine the competitive index (CI). As shown in Figure 3, the CI is 1 at 0 dpi, showing that the initial concentrations of *B. glumae* (*pGFP-TIR*) and *P. fluorescens* were the same. Because the input corresponds to the number of bacteria at 0 dpi, the value of the CI is directly related to the ratios of *B. glumae* (*pGFP-TIR*)/*P. fluorescens.* Thus, the CI of 0.25 at 3 dpi indicates that the numbers of *B. glumae* (*pGFP-TIR*) are higher than those of *P. fluorescens*. At 5 dpi, the competition index is -1.57 suggesting that *B. glumae* is out-competing *P. fluorescens* (*pGFP-TIR*) since a negative competition index indicates the presence of more *B. glumae* than *P. fluorescens*.

Isolation of cell-free fractions from *P. fluorescens* and *B. cenocepacia* with antagonistic activity against *B. glumae*

The growth inhibition of *B. glumae* caused by *P. fluorescens* and *B. cenocepacia in vitro* (Chapter 2), led to the hypothesis that these bacteria release compounds that inhibit the growth of *B. glumae*. To test this hypothesis two different methods were used to generate cell-free preparations with antagonistic activity against *B. glumae*. The first method used agar plugs obtained from the zones of inhibition and the second method used secreted and concentrated fractions of bacterial cultures supernatants.

Amending KB broth with fractions obtained from agar plugs corresponding to zones of inhibition caused by *P. fluorescens* and *B. cenocepacia,* reduced the growth of *B. glumae* as

indicated by reduced turbidity (Figure 4A). In agreement with this results, the numbers of *B. glumae* grown on KB containing agar plugs from LB plates, not exposed to either *P. fluorescens* or *B. cenocepacia* reached 10^7 CFU/ml. However, *B. glumae* grown in KB broth amended with a cell-free preparation derived from *P. fluorescens* reached 10^4 CFU/ml and *B. glumae* grown in KB broth supplemented with a cell-free preparation derived from *B. cenocepacia* reached 10^5 CFU/ml (Figure 4B) indicating that the cell-free preparations derived from *P. fluorescens* and *B. cenocepacia* reduced growth of *B. glumae* by 10000-fold and 1,000-fold, respectively.

Using secreted supernatants that were further lyophilized to concentrate them while preserving biological activity showed a similar pattern of growth inhibition albeit to a lesser extent. *B. glumae* grown in KB broth amended with lyophilized LB reached populations higher than 10^7CFU/ml, whereas *B. glumae* grown in KB broth amended with lyophilized secreted fractions from *P. fluorescens* and *B. cenocepacia* reached numbers higher than 10^6CFU/ml (Figure 5).

Secreted fractions from *P. fluorescens* and *B. cenocepacia* reduce disease symptoms caused by *B. glumae*

To determine if lyophilized secreted fractions from *P. fluorescens* and *B. cenocepacia* have antagonistic activity against *B. glumae in planta,* lyophilized fractions were added to *B. glumae* inoculum and injected into rice plants. All plants inoculated in this experiment developed lesions, however plants inoculated with *B. glumae* alone or with lyophilized LB had larger stem lesions (Figure 6A) that averaged 126.25mm and 115.5 mm, respectively (Figure 6B). In contrast plants inoculated with *B. glumae* that contained lyophilized secreted fractions from *P. fluorescens* or *B. cenocepacia* had average lesion lengths of 59.25 and 93.25mm, respectively (Figure 6B), demonstrating that the antagonistic activity against *B. glumae* was preserved in the lyophilized secreted fractions.

Discussion

B. cenocepacia and *P. fluorescens* were found to inhibit the growth of *B. glumae in vitro* and reduce symptoms of panicle blight when co-inoculated with *B. glumae* (Chapter 2). To facilitate the use of this finding to control panicle blight, this study showed that the antagonistic activity associated was preserved in cell-free preparations derived from *B. cenocepacia* and *P. fluorescens.* Isolating fractions with antagonistic activity from the agar has the advantage that the inhibitory compound (s) can diffuse into any liquid including water and preparations in water will facilitate future experiments to chemically characterize the compound (s) associated with that activity. Isolating culture supernatants for further lyophilization has the advantage that allows optimization of the amounts needed to achieve the desirable activity. While this work only used one concentration (0.01 g/ml), future work will determine optimal concentrations to reduce disease symptoms and *B. glumae* populations in the plant.

Altogether these findings supported one of the hypotheses that *P. fluorescens* and *B. cenocepacia* use antibiosis as a mechanism to inhibit growth of *B. glumae* with the concomitant effects on disease development. *Pseudomonas* species are known to produce diverse groups of antibiotics including phloroglucinols, phenazines, pyoluteorin, pyrrolnitrin, HNC (hydrogen cyanide) and lipopeptides (Haas and Keel, 2003), several of them with demonstrated activity controlling plant diseases. For example, the phenazine antibiotic phenazine-1-carboxylate produced by *Pseudomonas fluorescens* 2-75 (NRRL B-15132) was found to control *Gaeumannomyces graminis* var, tritici, the causal agent of take-all of wheat (Thomashow and Weller, 1988). *Pseudomonas sp.* also produce siderophores that take up the available iron required for microbial growth and that effect has been observed with *Pseudomonas* sp. Rh323 against *Xanthomonas oryzae* pv. oryzae and *Pseudomonas fluorescens* strain B10 against *Fusarium oxysporum* f. sp. lini and *Gaeumannomyces graminis* var tritici (Kloepper et al., 1980; Yasmin et al., 2016). Similarly, some *Burkholderia sp*. also produce antibiotics. For example, *B. cepacia* can also produce pyrrolnitrin which is a class of broad spectrum antibiotic and

antifungal compounds (Raaijmakers et al., 2002; El-Banna and Winkelmann, 1998). It is possible that the growth inhibition of *B. glumae* is associated with antibiotics or siderophores previously characterized or with new compounds. Future chemical work isolating and characterizing those compounds will determine that.

This work also sought to determine whether competition could be another mechanism associated with the antagonistic activities of *P. fluorescens* and *B. cenocepacia* against *B. glumae*. The higher growth rate of *P. fluorescens* and *B. cenocepacia* in comparison with the growth rate of *B. glumae in vitro,* suggests that these bacteria utilize nutrients from the media faster than *B. glumae,* therefore limiting *B. glumae* growth. However, direct competition experiments *in vitro* and *in planta* to evaluate the growth of *B. glumae* in the presence of *P. fluorescens* did not support the mechanism of competition. *In vitro* experiments showed that the numbers of *B. glumae* were the same when grown alone or with *P. fluorescens*. The competition experiments *in planta* that included measuring the competitive index showed that *B. glumae* out-competes *P. fluorescens.* These results are consistent with the results obtained after testing persistence of *P. fluorescens in planta.* As a non-pathogen *P. fluorescens* is unable to use plants to grow while as a true pathogen, *B. glumae* is able to multiple and reach higher numbers.

The identification of two bacterial strains with antagonistic activity against *B. glumae* and the discovery that cell- free secreted fractions preserve that activity is significant as it will pave the way to further analyses of those fractions to identify the compound(s) responsible for that activity which in turn will facilitate the use of these compounds as a possible solution to control *B. glumae* and mitigate the damages caused by Bacterial Panicle Blight.

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Appendix

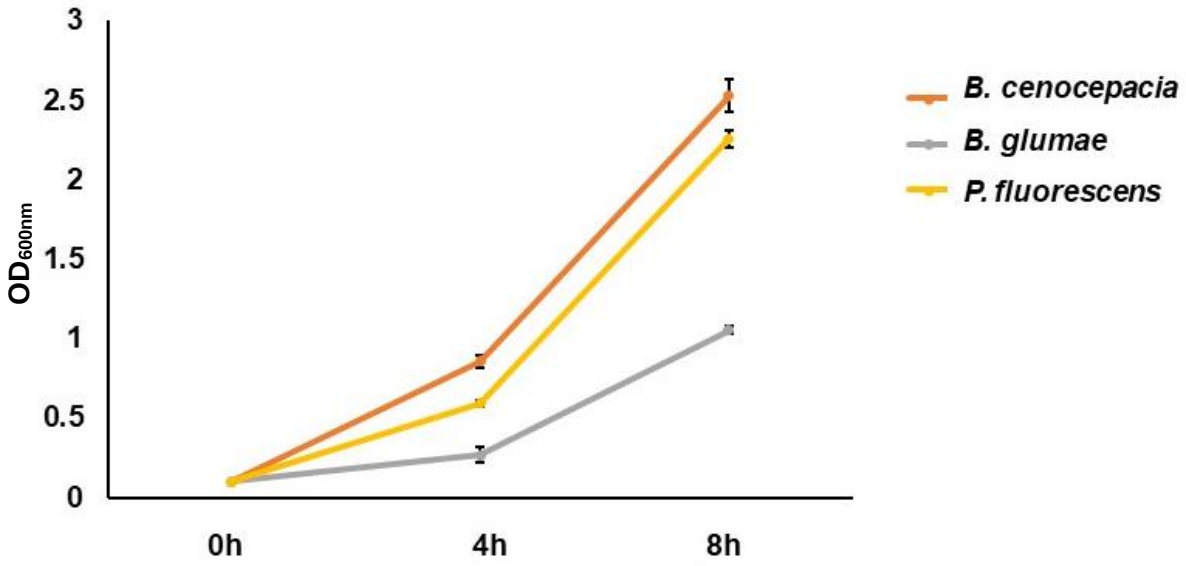

Figure 3.1. Comparison of bacterial growth of *B. cenocepacia*, *B. glumae* and *P. fluorescens in vitro.* Overnight cultures of *B. cenocepacia, B. glumae* and *P. fluorescens* were diluted to OD600=0.2 in 100 mL of KB and incubated in a 30^{o}C shaker. Aliquots were taken at 0h, 4h and 8h and bacterial growth assessed by measuring absorbance at OD_{600}. Data was analyzed using ANOVA statistical analysis with P-value <0.001.

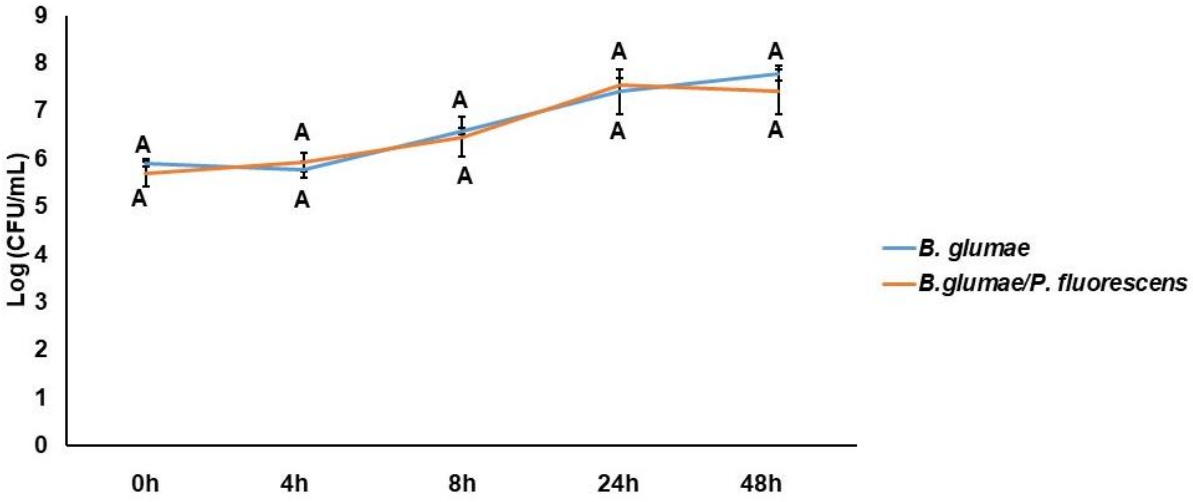

Figure 3.2. Competition assay between *B. glumae* and *P. fluorescens in vitro.* *B. glumae* (*pGFP-TIR*) was grown in 100 mL of KB broth alone or in combination with *P. fluorescences* (ration 10:1). Single and mixed cultures were incubated in 30^{o}C by shaking. Aliquots from single and mixed cultures were taken at 0h, 4h, 8h, 24h and 48h, serially diluted and plated on KB containing gentamycin to specifically enumerate *B. glumae* (*pGFP-TIR*) bacteria. The graph represents bacterial numbers of *B. glumae* (*pGFP-TIR*) alone and when growth with *P. fluorescens.* For each time point, the growth of *B. glumae* (*pGFP-TIR*) grown alone was compared with that of *B. glumae* (*pGFP-TIR*) grown with *P. fluorescens* and analyzed using ANOVA statistical analysis with P- value = 0.6310 at 0h, 0.0365 at 4h, 0.514 at 8h, 0.8211 at 24h and 0.3696 at 48h.

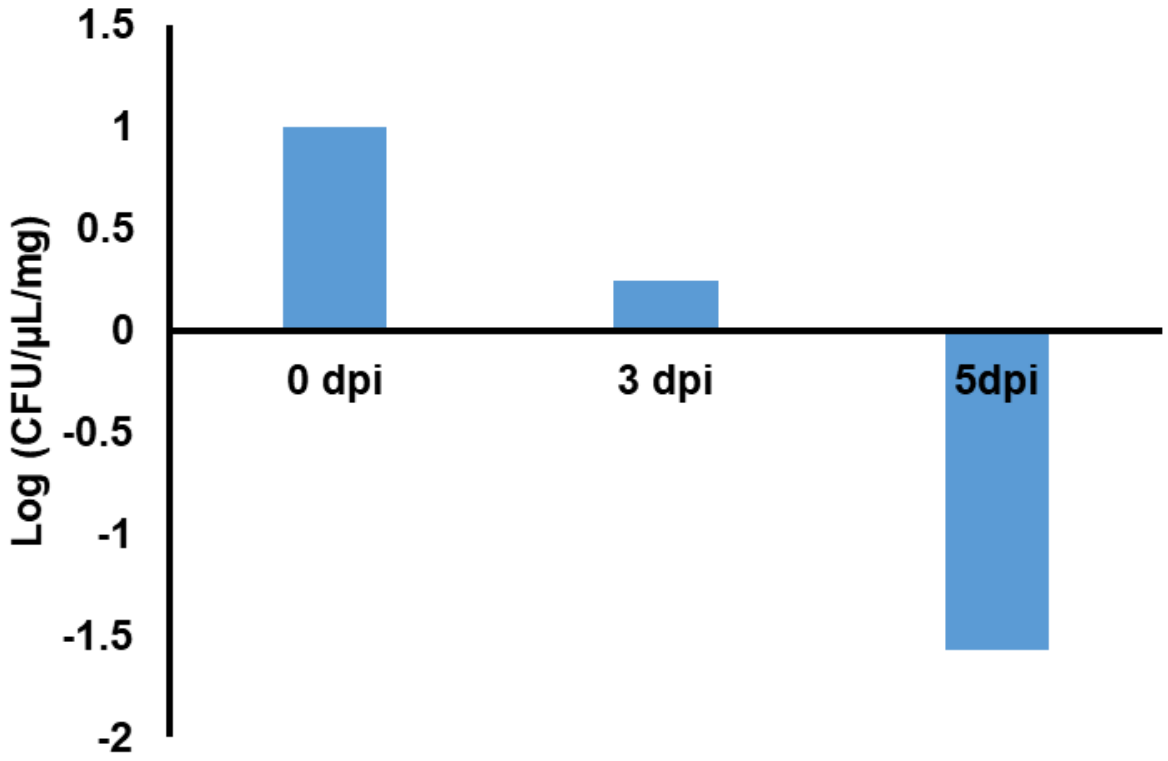

Figure 3.3. Competition assay in vivo and competitive index between *B. glumae* and *P. fluorescens*. Four- week-old rice plants, cultivar Wells were inoculated with *B. glumae* (*pGFP-TIR*) alone or mixed with *P. fluorescens*. Bacterial inoculums were adjusted to OD_{600}= 0.125. Plants were ground, serially diluted and plated at 0, 3 and 5 dpi on KB plates and KB containing gentamycin. The numbers of *B. glumae* (*pGFP-TIR*) were counted on KB plates containing gentamycin. The numbers of *P. fluorescens* were calculated by subtracting the numbers of *B. glume* (*pGFP-TIR*) from the total number of colonies obtained in KB alone. Bars represent the competitive index for each time point.

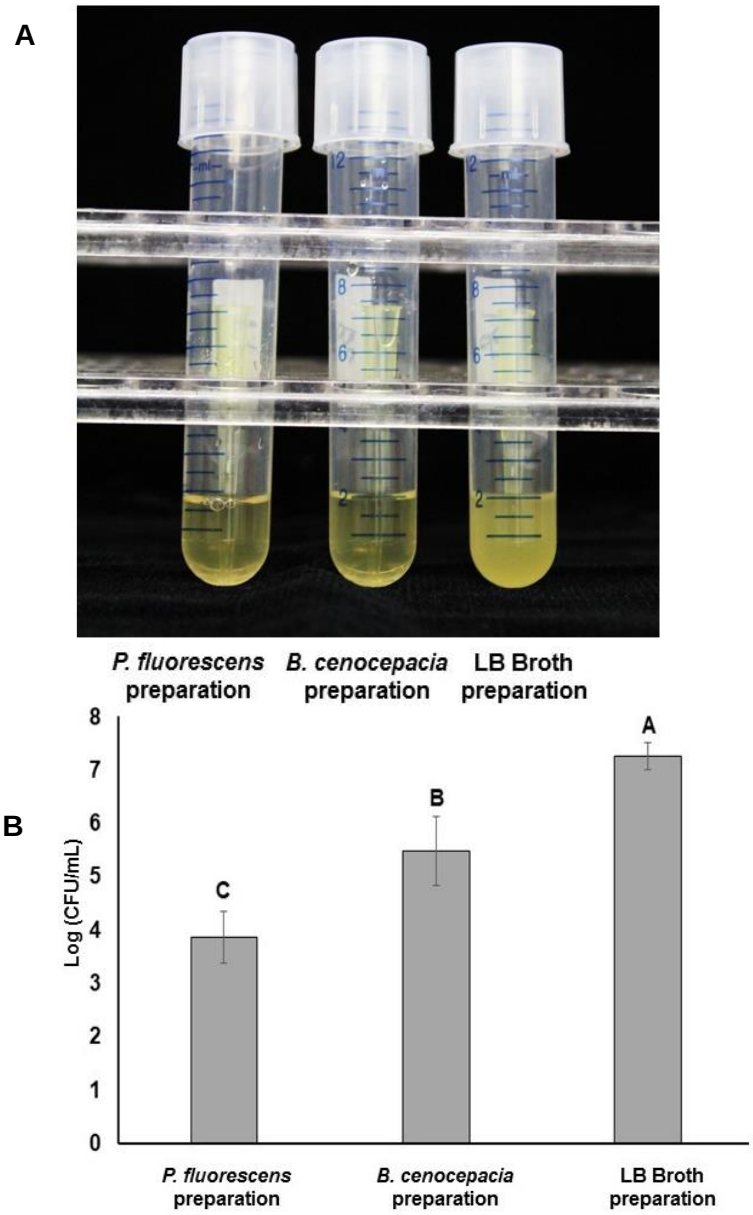

Figure 3.4. *B. glumae* grown in the presence of filter sterilized KB broth which was incubated with agar plugs from zone of inhibition plates containing *B. glumae* and *P. fluorescens* or *B. cenocepacia.* Agar plugs were taken from LB agar not exposed to *P. fluorescens* or *B. cenocepacia* and from the zones of inhibition caused by *B. cenocepacia* or *P. fluorescens* on *B. glumae* plates. Agar plugs were shaken in 3mL KB broth for 24h at 30^{o}C to allow diffusion of inhibitory compounds into the KB. After incubation, these preparations were filter-sterilized. One milliliter of each preparation was mixed with 1 ml of KB and used to grow a single colony of *B. glumae*. Tubes were shaken at 30^{o}C for 24h to qualitatively evaluate bacterial growth (A). One hundred microliters of bacterial growth was serially diluted and plated to count populations of *B. glumae* (B). Bars represent the growth of *B. glumae* in the presence of the different preparations and the comparison among preparations was analyzed by ANOVA statistical analysis with P- value = 0.0153.

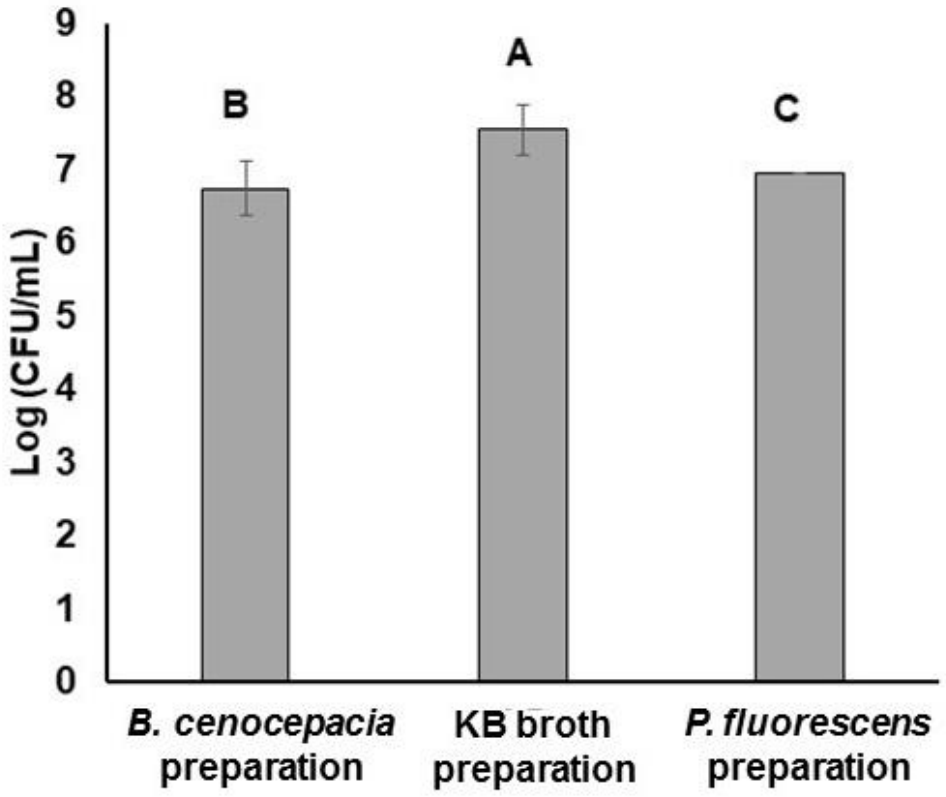

Figure 3.5. Lyophilized supernatants from *P. fluorescens* and *B. cenocepacia* reduce growth of *B. glumae.* Lyophilized LB broth, *B. cenocepacia* supernatant and *P. fluorescent* supernatants were diluted to 0.01g/mL. This solution was mixed in a 1:1 ratio (V/V) with KB broth and filter-sterilized. A single *B. glumae* colony was added to these tubes to inoculate them and grown for 24 h at 30^{o}C with agitation. At 24h, aliquots were taken, serially diluted and plated to count the number of bacteria in CFUml on KB plates. Bars represent the growth of *B. glumae* in the presence of the different preparations and the comparison among preparations was analyzed by ANOVA statistical analysis with P- value= 0.3722.

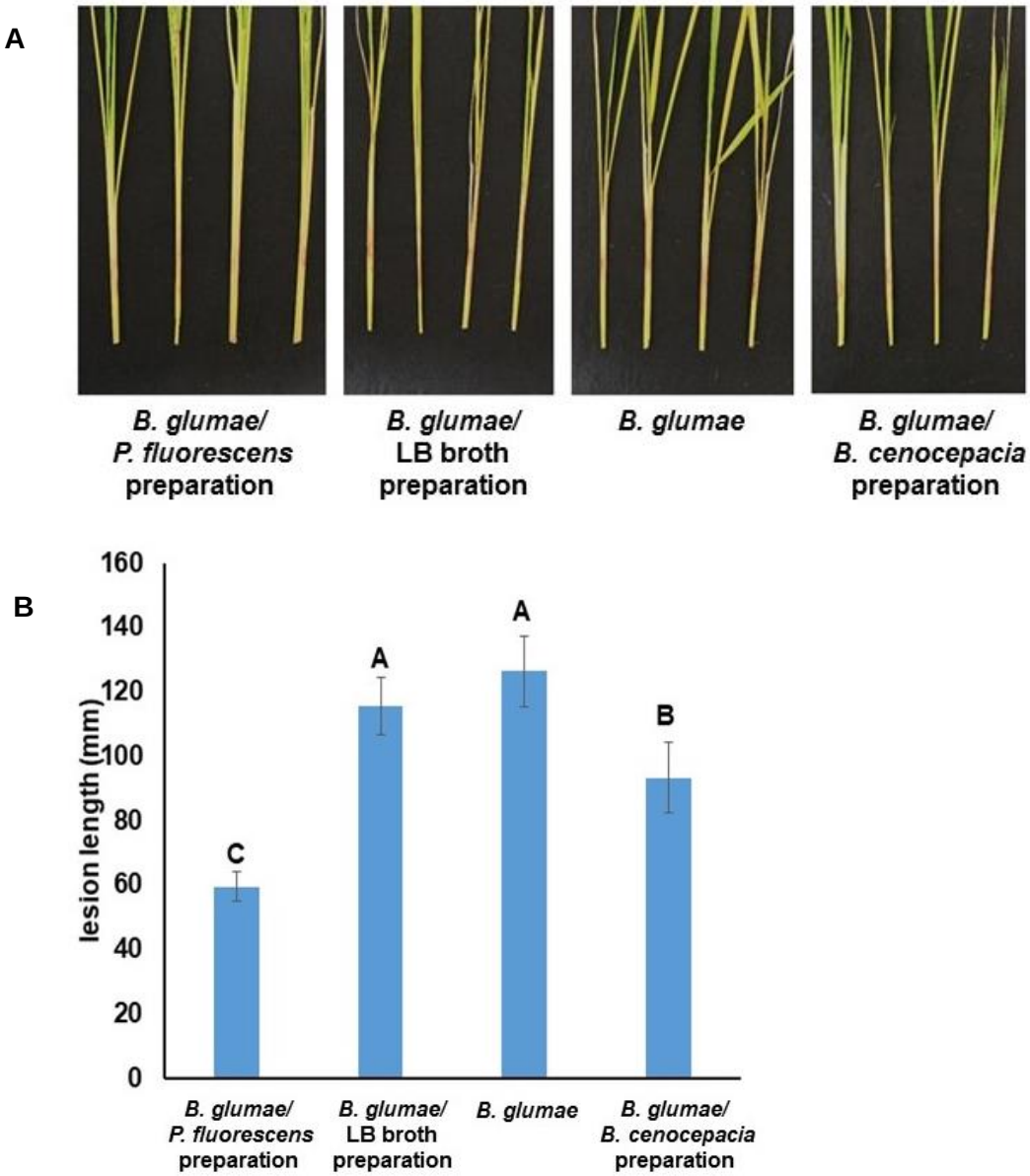

Figure 3.6. Lyophilized supernatants from *P. fluorescens* and *B. cenocepacia* reduced disease symptoms caused by *B. glumae*. Eight- week- old rice plants cultivar Wells plants were injected with 20µL *B. glumae* at OD_{600}= 0.125µL or *B. glumae* containing 0.1g/mL of lyophilized supernatants from either *P. fluorescens* or *B. cenocepacia* cultures or lyophilized LB broth. Disease symptoms were observed at 8 days after inoculation (A). Bars in (B) show the length of the lesions. The differences among lesion sizes were analyzed using ANOVA statistical analysis, P- value= 0.0003.

Conclusion

This research was performed to identify a biological control agent against the emerging rice disease Bacterial Panicle Blight of rice, caused by the bacteria *Burkholderia glumae*. Two potential sources of biological control agents were mined, Jupiter rice associated bacteria, and the Rojas Lab collection. The hypothesis that the composition of rice associated bacteria from moderately resistant Jupiter and susceptible Bengal would be different was supported by the experiments and six bacterial strains unique to Jupiter were tested for their inhibitory activity against *B. glumae*. Though some Jupiter rice associated bacteria did show small amounts of inhibition against the pathogen, the effect was not enough to warrant further exploration as biological control agents though it is possible that they contribute to the moderate resistance of the rice cultivar, which was our second hypothesis. Two bacterial strains, *Pseudomonas fluorescens* and *Burkholderia cenocepacia*, from the Rojas Lab collection showed very promising results and were thus pursued. These bacterial strains were able control *B. glumae in vitro* and *in planta* suggesting they would be successful at controlling the disease in the field. Unfortunately these bacterial strains were not able to persist well *in planta* and B. cenocepacia, being an opportunistic human pathogen, would not be safe to use as a biological control treatment in the field. However when the mechanisms of control for these bacteria were explored they gleaned promising results. When the hypothesis that these biological control bacteria used competition as their main control agent was explored it was not supported by the data, but when the hypothesis of antibiosis was explored it was found that supernatant containing secreted fractions from the control agents but no live cells were still able to inhibit the pathogen *in vitro* and *in planta*. This is promising as it shows the potential for a control method against the disease to be developed without having to use bacterial strains which do not persist well in the field and are potentially harmful to human health.